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GLYPICAN-1 PROTEOLIPOSOMES ENHANCE GROWTH FACTOR ACTIVITY FOR THERAPEUTIC ANGIOGENESIS

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GLYPICAN-1 PROTEOLIPOSOMES ENHANCE GROWTH FACTOR ACTIVITY FOR THERAPEUTIC ANGIOGENESIS

by

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Dedication

To my parents

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I would like to thank my family for being supportive and encouraging over the last four years. I would especially like to thank my parents for listening to me when I was having a hard time in the lab and for being excited for me when things were going well. Even though it was decently embarrassing, I will never forget when they snuck into my very first talk at the Biomedical Engineering Society meeting in Seattle with my grandparents. Also I am willing to bet that the entirety of my success is based on the hooked on phonics chart they made me memorize as soon as I learned how to talk ("Anthony, 'S' 'H' makes the sound of?"). If there is anyone who is more excited about my completion of this program than I am, it is my family, you are all the best.

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Glypican-1 Proteoliposomes Enhance Growth Factor Activity FOR Therapeutic Angiogenesis

Anthony Joseph Monteforte IV, Ph.D.

The University of Texas at Austin, 2016

Supervisor: Aaron B. Baker

Peripheral arterial disease affects more than 27 million patients in the United States [1]. PAD can lead to peripheral limb ischemia and result in non-healing foot ulcers. Currently, surgical therapies such a stenting, grafting, and bypass, exist for treatment of ischemia, but these treatments are prone to long-term failure. These treatments also only successfully yield beneficial results about 25% of the time [2]. Alternatively, regenerative therapies that stimulate the growth of new vasculature have great potential for treating peripheral and myocardial ischemia. Growth factor based therapies that induce neovascularization have shown promising results in animal models, but have limited success in clinical trials [3]. This discrepancy is most likely a result of growth factor resistance brought on by diseases that lead to peripheral vascular disease [4]. Here, we have developed a new method for enhancing the activity of growth factors in growth factor resistant disease states such as diabetes and hyperlipidemia. Our novel method delivers the growth factor co-receptor glypican-1 embedded in a liposomal carrier to create a glypican-1 proteoliposome (a "glypisome"). By co-delivering the co-receptor glypican-1 along with the growth factor, we hope to overcome growth factor resistance associated with long-term disease. Here we optimize glypisome composition to

maximize angiogenic response when co-delivered with growth factors, through the use of *in vitro* endothelial assays and explore what mechanisms bring about this change in activity. Then we determine therapeutic potential of co-delivering glypisomes with growth factors *in vivo* by assessing neovascularization in healthy and disease mouse models of ischemia. We also explore overcoming disease mediated growth factor resistance by delivering glioblastoma-derived exosomes as a naturally occurring alternative to the glypisomes that we have developed. We test these exosomes in both *in vitro* endothelial assays and *in vivo* with a mouse hind limb ischemia model. We demonstrate that delivering these co-receptors in conjunction with the growth factor will allow us to overcome the disease phenotype and lead to a viable growth factor therapy for peripheral arterial disease.

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Chapter 1: Introduction

1.1 Motivation

Peripheral arterial disease (PAD) affects over 200 million people worldwide and is estimated to afflict approximately 16% of the general population over 65 years of age. Severe PAD has serious consequences for patients including the formation of non-healing ulcers, pain from intermittent claudication and, ultimately, increased risk for limb amputation. The current clinical treatments for PAD include surgical revascularization with bypass grafting or endartectomy, or percutaneous interventions such as angioplasty, stenting and atherectomy. However, these procedures cannot be performed in a significant portion of patients and many do not respond to these therapies [5]. An alternative approach for treating ischemic disease is to stimulate the body to create new vasculature to restore blood flow through its own regenerative processes. Several approaches have been explored to this end including the delivery of progenitor cells [6], viral vectors to express growth factor/angiogenic transcription factor genes [6-15] or through the delivery of growth factors [7, 16, 17]. Growth factors as protein therapeutics for ischemia have potential advantages from regulatory, production and delivery perspectives. However, in practice angiogenic growth factor therapies, both through delivered proteins and genes, have led to disappointing results in clinical trials [18]. Thus, while the concept of therapeutic angiogenesis has great promise, there are no current treatments that are capable of stimulating neovascularization in the context of human ischemic disease.

The presence of diabetes increases the risk of developing PAD by two-fold and increases the rate of progression of the disease [5]. Diabetic patients are 5-10 fold more likely to need limb amputation due to PAD [19]. The disease processes that drive development of ischemia may also induce disruptions in the pathways that are critical to

mounting an effective angiogenic response to growth factor therapy. Insulin resistance is a hallmark of diabetic disease and, similarly, long-term ischemic vascular disease in aged humans may also represent a state in which the body can no longer respond effectively to growth factors such as FGF-2 and VEGF in ischemic tissues. We have recently shown that many of the heparan sulfate proteoglycans (HSPGs) that serve as co-receptors for the FGF-2 and VEGF families of growth factors are expressed at lower levels in ob/ob mice than wild type (WT) mice [4]. In addition, heparanase expression is increased in cells treated with fatty acids and in animals on a high fat diet [20-23], in atherosclerotic plaques [23-25] and following stenting or vascular injury [26, 27]. Heparanase is an enzyme that can degrade the heparan sulfate chains on cell surface proteoglycans [28, 29], ultimately leading to enhanced surface shedding of these molecules and loss of their activity as co-receptors for growth factor signaling [30, 31]. Thus, many disease states and pathophysiological processes lead to the loss of HSPGs from the cell surface.

In this study, we have examined the expression of the cell surface proteoglycan glypican-1 in disease and explored its use as a therapeutic enhancer for angiogenic growth factor delivery. The glypicans are distinguished from other cell surface HSPGs by their linkage to the membrane through a glycosylphosphatidylinositol (GPI) anchor. This GPI linker enables the phospholipase mediated-shedding of glypicans and drives preferential localization of the protein in cholesterol-rich lipid rafts [32]. These properties allow glypicans to associate with calveolae [33], control endocytosis/recycling and transcellular transport [34-37], regulate the formation of morphogen gradients [38, 39] and facilitate growth factor cell signaling [40-43]. Glypican-1, in particular, is highly expressed in gliomas and their associated vasculature [44, 45]. A hallmark of gliomas is vigorous angiogenic response to the tumor that drives neovascularization through multiple mechanisms [34-37]. Glypican-1 has a key role in the growth, metastasis and

angiogenic properties of gliomas [44-47]. In addition, glypican-1 is the most prevalent member of the glypican family expressed in endothelial cells and the vascular system [48]. It can act as a co-receptor/modulator for many angiogenic factors including members of the FGF and VEGF growth factor families [44, 48-51]. Several studies also have shown that glypican-1 can stimulate cell cycle progression in endothelial cells [47, 52]. In this work, we show that glypican-1 is lost in the blood vessels of skin samples from human patients with type 2 diabetes. We developed a novel therapeutic that consists of glypican-1 embedded in a nanoliposomal carrier (a "glypisome") that can be locally delivered to ischemic tissues from an alginate gel to potentiate the angiogenic response to localized growth factor therapies in diseased tissues. We demonstrate that this therapeutic enhancer improves the *in vitro* activity of FGF-2 and VEGF in endothelial cells through multiple mechanisms. When locally delivered from an alginate gel, glypisomes also enhance the therapeutic potential of FGF-2 therapy leading to increased revascularization of ischemic limbs in wild type and diabetic mice.

Exosomes are nanoparticles that are secreted by cells into their environment and range from approximately 30 to 100 nm in diameter [53]. These secreted extracellular vesicles are formed initially by formation of invaginations in the endosomal membrane to create a multivesicular body (MVB)[54]. This process is in contrast to the that for microvesicles (200-1000 nm in diameter) that are formed by membrane shedding[55]. If true/exclusive exosomes are released through multivesicular endosomal fusion with the plasma membrane and are found in many biofluids. Extracellular vesicles were initially discovered in several research fields with varying nomenclature. These secreted vesicles have now been recognized as mediators of cell-cell communication, capable of delivering molecules between distant cells [56]. Exosomes, in particular are known to have high levels of tetraspanins, trafficking/export-related molecules and heat shock proteins[57-

60]. In addition, exosomes contain proteins, microRNA (miRNA) and, in some cases, double stranded DNA [61, 62]. Exosomes also carry bioactive lipids including sphingomyelin, eicosanoids, cholesterol and ganglioside GM3 [63]. Extracellular vesicles including exosomes are efficiently taken up by cells in culture where they modify the target cells transcriptional and protein expression profiles.

In particular, exosomes from many cell types have been known to mediate angiogenesis [64-69]. Exosomes derived from mesenchymal stem cells have also been shown to induce angiogenesis and modulate revascularization in ischemia and wound healing [68, 69]. Glioblastoma is one of the most angiogenic tumors, induce an intense growth of surrounding blood vessels [70]. The exosomes secreted by glioblastoma cells prime endothelial cells to respond to hypoxic conditions with an angiogenic response [71]. Moreover, primary glioblastoma cells carry angiogenic miRNAs and proteins that facilitate angiogenic differentiation of endothelial cells [72]. Extracellular vesicles from glioblastoma cells have been shown to carry proteins that have both oncogenic and tumor suppressive activities including EGF receptors, PDGFR-A and PTEN [72-75]. Thus, while it is well supported that glioblastoma exosomes enhance angiogenesis it is less clear what role they play in tumor progression and metastasis. In addition, glioma-derived exosomes have immunomodulatory effects and drive macrophages towards the M2-phenotype [76]. Macrophages polarized towards the M2-phenotype secrete VEGF and promote angiogenesis [77, 78].

Exosomes are an emerging therapeutic strategy and have been explored as direct treatments for disease and drug carriers [79-82]. Exosomes from mesenchymal stem cells have generated significant interest and have been used to induce angiogenesis in wound healing and in ischemia [69, 83]. In addition, mesenchymal stem cell exosomes have improved the recovery following myocardial infarction and enhance cardiac

regeneration [84, 85]. In this study, we aimed to examine using glioblastoma exosomes as therapeutics for enhancing revascularization in peripheral limb ischemia. Glioblastoma is one the most highly vascularized solid tumors and produces an intense angiogenic response. Moreover, the exosomes produced by these cells have been linked to growth promoting signals in endothelial cells.[86] Here, we explored whether this powerful aspect of tumor biology could be harnessed to enhance angiogenic therapies for patients with peripheral vascular disease.

1.2 DISSERTATION ROADMAP

This dissertation is laid out such that chapter one is an introduction to the motivation for the work. Chapter two contains the relevant background for the projects described in the subsequent chapters. Chapter three describes the fabrication, characterization and optimization of the glypisomes using *in vitro* assays of angiogenesis. Chapter four describes *in vivo* analysis of the angiogenic potential of the glypisomes as a treatment for peripheral vascular disease using a murine hind limb ischemia model. Chapter five details the therapeutic potential of a naturally occurring glypisome-like-particle, glioblastoma exosomes, for therapeutic angiogenesis in peripheral limb ischemia. This chapter describes the isolation and characterization as well as in vitro and in vivo validation of these exosomes as a potential therapeutic. Finally, chapter six contains the conclusions from this work and future directions to further explore these therapies.

Chapter 2: Background

2.1 Peripheral Vascular disease

Peripheral arterial disease (PAD) affects more than 27 million patients in the United States [1]. It is characterized by arterial narrowing due to plaque buildup, reducing blood flow in the affected tissues. This leads to poor oxygenation and a buildup of metabolic by products. PAD can lead to peripheral limb ischemia and result in non-healing foot ulcers, and is associated with increased risk of myocardial infarction, critical limb ischemia, stroke, and death due to a cardiovascular related event. Patients with critical limb ischemia, which is characterized by resting pain in the lower extremities, have a 25% chance of amputation and/or cardiovascular related mortality within the year of diagnosis. Five years after developing critical limb ischemia the likelihood of cardiovascular mortality leaps to 60% [87]. Increased risk for developing PAD is associated with smoking, hypertension, diabetes, high cholesterol, sedentary behavior and obesity [88, 89]. There are several methods for diagnosing PAD; ankle-brachial index, doppler ultrasound, magnetic resonance angiogram, and arteriogram may be employed to detect the disease. Skin perfusion measurements are also an indicator of critical limb ischemia [90].

Currently, surgical therapies such a stenting, grafting, and bypass, exist for treatment of ischemia, but these treatments are prone to long-term failure and are not always an option for patients due to other comorbidities [2]. These treatments are also only successfully yield beneficial results about 25% of the time [2]. Alternatively, regenerative therapies that stimulate the growth of new vasculature have great potential for treating peripheral and myocardial ischemia. Growth factor based therapies that

induce neovascularization have shown promising results in animal models, but have limited success in clinical trials [3]. This discrepancy is most likely a result of growth factor resistance associated brought on by diseases that lead to peripheral vascular disease [4].

2.2 ANGIOGENESIS

Angiogenesis is the formation of new vasculature from by branching off of the existing vascular network. Therapeutic angiogenesis can be achieved by introducing stem cells or growth factors to stimulate new vascular formation. This leads to an increase in blood flow in the area. There are several ways in which this can occur; growth factors can be upregulated by gene therapy or by local delivery of the growth factors. Growth factors that are targeted to induce angiogenesis are, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF) [91-95]. There are several concerns associated with a gene therapy approach, such as controlling gene integration in to the target cells. Also due to the complexity of angiogenesis it is possible that a single gene target may not be effective to overcome challenges posed by the disease.

Stem cell therapies have also been explored. There is evidence that injecting mesenchymal stem cells into ischemic tissue leads to angiogenesis in this tissue [96-99]. It is thought that this result is due to both direct incorporation of these cells into the vascular network, and by the proangiogenic signals that these cells produce. Stem cell therapies are particularly attractive as they could use an autologous source for these cells. While attractive, stem cell therapies still are not quite well developed enough for effective and controlled treatments.

2.3 GLYPICAN-1

Glypican-1 is a cell surface heparan sulfate proteoglycan (HSPG). HSPGs are cell surface proteins that consist of a core protein with covalently attached heparan sulfate chains or glycosaminoglycans. The glypicans are distinguished from other cell surface HSPGs by their linkage to the membrane through a glycosylphosphatidylinositol (GPI) anchor. This GPI linker enables the phospholipase mediated-shedding of glypicans and drives preferential localization of the protein in cholesterol-rich lipid rafts [32]. These properties allow glypicans to associate with calveolae [100], control endocytosis/recycling and transcellular transport [101-104], regulate the formation of morphogen gradients [105, 106] and facilitate growth factor cell signaling [107-110]. Glypican-1, in particular, is highly expressed in gliomas and their associated vasculature [111, 112]. A hallmark of gliomas is vigorous angiogenic response to the tumor that drives neovascularization through multiple mechanisms [101-104]. Glypican-1 has a key role in the growth, metastasis and angiogenic properties of gliomas [111, 113, 114]. Glypican-1 has also been used to identify cancer cell derived exosomes[115]. It can act as a co-receptor/modulator for many angiogenic factors including members of the FGF and VEGF growth factor families [111, 116-118].

2.4 EXOSOMES

Exosomes are extracellular vesicles released by cells into their environment that range from 30-100 nm in diameter. They are released via multivesicular endosomal fusion with the plasma membrane [119, 120] and are found in many different types of biofluids. They contain proteins, microRNA, and DNA. Exosomes have been shown to facilitate intracellular signaling by delivering the protein and RNA [119, 121, 122]. We have decided to focus on glioblastoma exosomes because glioblastoma is one of the most

vascular solid tumors [123]. Exosomes from glioblastoma have also been shown to release the pro-angiogenic signal miR-124 [124].

2.5 MOUSE MODELS

In these studies, C57Bl/6 mice were used as a healthy model or wild type (WT). C57Bl/6 mice are the most widely used strain of mice and is one of the earliest species to have its genome sequenced. To model the disease state we will use B6.Cg-Lep^{ob}/J (ob/ob) or leptin deficient mice, and originate from the C57Bl/6 strain. These mice are leptin deficient and characterized by obesity, hyperglycemia and hyperlipidemia. The obesity in these mice is characterized by both an increase in size and number of adipocytes. While hyperphagia leads to obesity in these mice, they will gain excess fat deposits even on a restricted diet. The ob/ob mice have been shown to have glucose intolerance, elevated plasma insulin and impaired wound healing and are a good model for diabetes. Our lab has also shown that these mice exhibit disease induced growth-factor resistance to FGF-2 and VEGF [4]. For these reasons we have chosen them as our disease model.

Chapter 3: Development of Glypican-1 Proteoliposomes

3.1 Introduction

Peripheral arterial disease (PAD) affects over 200 million people worldwide and is estimated to afflict approximately 16% of the general population over 65 years of age. Severe PAD has serious consequences for patients including the formation of non-healing ulcers, pain from intermittent claudication and, ultimately, increased risk for limb amputation. The current clinical treatments for PAD include surgical revascularization with bypass grafting or endartectomy, or percutaneous interventions such as angioplasty, stenting and atherectomy. However, these procedures cannot be performed in a significant portion of patients and many do not respond to these therapies [5]. An alternative approach for treating ischemic disease is to stimulate the body to create new vasculature to restore blood flow through its own regenerative processes. Several approaches have been explored to this end including the delivery of progenitor cells [6], viral vectors to express growth factor/angiogenic transcription factor genes [6-15] or through the delivery of growth factors [7, 16, 17]. Growth factors as protein therapeutics for ischemia have potential advantages from regulatory, production and delivery perspectives. However, in practice angiogenic growth factor therapies, both through delivered proteins and genes, have led to disappointing results in clinical trials [18]. Thus, while the concept of therapeutic angiogenesis has great promise, there are no current treatments that are capable of stimulating neovascularization in the context of human ischemic disease.

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likely to need limb amputation due to PAD [19]. The disease processes that drive development of ischemia may also induce disruptions in the pathways that are critical to mounting an effective angiogenic response to growth factor therapy. Insulin resistance is a hallmark of diabetic disease and, similarly, long-term ischemic vascular disease in aged humans may also represent a state in which the body can no longer respond effectively to growth factors such as FGF-2 and VEGF in ischemic tissues. We have recently shown that many of the heparan sulfate proteoglycans (HSPGs) that serve as co-receptors for the FGF-2 and VEGF families of growth factors are expressed at lower levels in ob/ob mice than wild type (WT) mice [4]. In addition, heparanase expression is increased in cells treated with fatty acids and in animals on a high fat diet [20-23], in atherosclerotic plaques [23-25] and following stenting or vascular injury [26, 27]. Heparanase is an enzyme that can degrade the heparan sulfate chains on cell surface proteoglycans [28, 29], ultimately leading to enhanced surface shedding of these molecules and loss of their activity as co-receptors for growth factor signaling [30, 31]. Thus, many disease states and pathophysiological processes lead to the loss of HSPGs from the cell surface.

In this study, we have examined the expression of the cell surface proteoglycan glypican-1 in disease and explored its use as a therapeutic enhancer for angiogenic growth factor delivery. The glypicans are distinguished from other cell surface HSPGs by their linkage to the membrane through a glycosylphosphatidylinositol (GPI) anchor. This GPI linker enables the phospholipase mediated-shedding of glypicans and drives preferential localization of the protein in cholesterol-rich lipid rafts [32]. These properties allow glypicans to associate with calveolae [33], control endocytosis/recycling and transcellular transport [34-37], regulate the formation of morphogen gradients [38, 39] and facilitate growth factor cell signaling [40-43]. Glypican-1, in particular, is highly expressed in gliomas and their associated vasculature [44, 45]. A hallmark of gliomas is

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3.2 Materials and Methods

3.2.1 Human Tissue, Immunostaining

Human skin samples were obtained from the Glasgow Caledonian University Skin Research Tissue Bank, Glasgow UK. The tissue bank has NHS research ethics to supply human skin for research (REC REF: 11/S1402/2). Samples were formalin fixed and embedded in paraffin following standard procedures prior to sectioning. Six skin samples were collected from non-diabetic patients and seven skin samples from those with type 2 diabetes. Human skin tissue samples were cut with a microtome to obtain 6

μm thick sections were then stained using hematoxylin and eosin for overall morphology. The sections were deparaffinized and treated for 3 hours with antigen retrieval solution (Dako) at 80°C. The sections were cooled to room temperature and blocked with 20% fetal bovine serum for 45 minutes and then immunostained with the Envision+ Dual Link Kit (Dako) using a 1:100 dilution of primary antibody to PECAM-1 (Millipore) or a 1:500 dilution of primary antibody to glypican-1 (Thermo Scientific). Following staining, the samples were imaged using a bright field microscope (Meiji). For human skin samples, we defined DAB intensity for positive staining and counted the number of cells positive for glypican-1 staining in the arterioles in the skin.

3.2.2 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in MCDB-131 media with 7.5% FBS, EGM-2 supplements (Lonza), L-glutamine and antibiotics. Endothelial cells that were used for the experiments did not exceed passage six. HeLa cells (ATCC) were cultured in high glucose DMEM with 10% FBS, L-glutamine and antibiotics. For lentiviral production, HEK293T cells were cultured in DMEM with sodium pyruvate, 10% FBS, L-glutamine and antibiotics.

3.2.3 Preparation of Glypisomes

For the production of recombinant glypican-1, HeLa cells were transduced with a lentiviral vector with constitutive expression of 6x-His tagged glypican-1. Viruses were produced in HEK 293T cells using a human lentiviral packaging system according to the manufacturer's instructions (Genecopoeia). The cells were then selected with puromycin to create a stable cell line. The cells were lysed in a buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 2 mM PMSF, 50 mM NaF, and protease inhibitors (Roche) at room temperature. Glypican-1 was then

isolated using a Ni Sepharose column (His SpinTrap Kit; GE Healthcare). The buffer was exchanged and protein concentrated using Centriprep concentrators (Millipore). The final working concentration of protein was found to be 61 μg/ml using a micro BCA Protein Assay kit (Thermo Scientific). Purity of the protein was confirmed by SDS-PAGE followed silver staining of the gel (Figure 3.1). We then mixed lipid stock solutions (10 mg/ml in chloroform) of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol and sphingomyelin in a volumetric ratio of 2:1:1:1, respectively, in a round bottom flask. The solvent was removed using rotary evaporation and the lipid film further dried under argon gas. The film was resuspended in HEPES buffer by vortexing, sonication and three cycles of freeze thawing (total lipid concentration of 12.3 mM). The lipid solution was then extruded through a 400 nm polycarbonate filter membrane to create liposomes. The liposome solution was mixed to varying volumes of glypican-1 (61 μg/ml) and 1% n-octyl-β-D-glucopyranoside. The detergent was extracted to allow glypican-1 incorporation into the liposome membrane through progressive dilution, dialysis and zeolite treatment. Every 30 minutes the concentration of the solution was reduced 10% by adding PBS to a final concentration of 40% of the original solution. The excess protein and detergent were then removed extensive dialysis and the final solution treated with Biobeads (SM-2; Bio-Rad) to remove any residual detergent. The final concentration of the glypisomes contained 664 μg/ml of the lipid solution and 19.5 μg/ml of the glypican-1 at the end of this process.

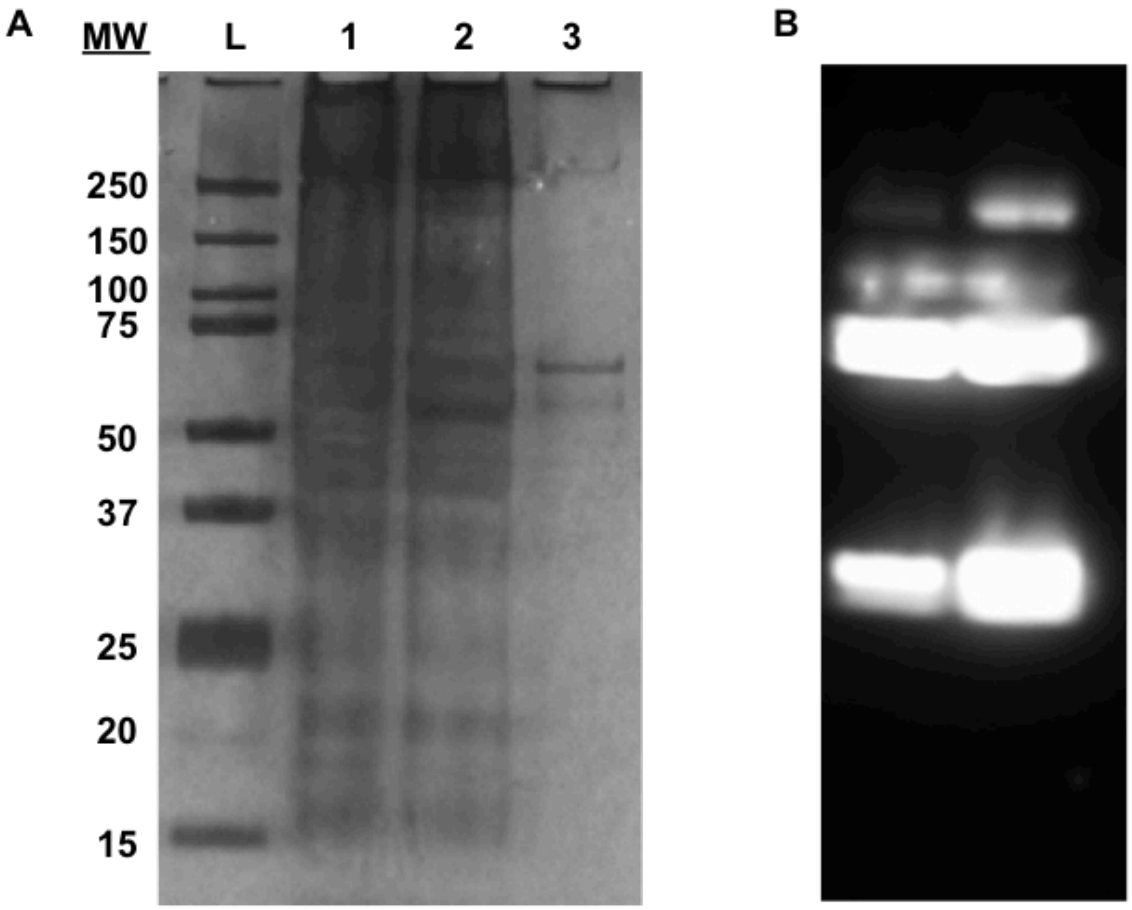

Figure 3.1 Isolation of recombinant glypican-1. (A) Silver stained gel of isolated glypican-1. L: molecular weight (MW) ladder labeled in kDa. Lane 1: whole lysate from HeLa cells. Lane 2: Whole lysates from glypican-1 over-expressing HeLa cells. Lane 3: isolated glypican-1. (B) Western blot for glypican-1 for the total sample (Lane 1) and the isolated glypican-1 (Lane 2).

3.2.3 Immunoblotting and Silver Staining

Following the various treatments, cells were lysed in a solution containing the following: 20 mM Tris at pH 8, 150 mM NaCl, 1% triton, 0.1% SDS, 2 mM sodium orthovanadate, 2 mM PMSF, 50 mM NaF, and protease inhibitors (Roche). For tissue samples, cryosections were collected, and the sections placed into the above lysis buffer and homogenized using a Tissuelyzer device (Qiagen). Lysates from cells or tissues were clarified with centrifugation, normalized for protein concentration and separated on Nupage Novex 10% Bis-Tris gels (Lifetech). Samples were then transferred using the iBlot System to a nitrocellulose membrane using the iBlot Gel Transfer Stacks (Life Technologies). The membrane was washed twice in phosphate buffered saline with 0.1% tween (PBST). The membranes were then incubated at 4°C overnight in a 1:500 dilution of glypican-1 antibody (Abcam), phospho-ERK, ERK, phosphor-AKT or AKT antibodies (Cell Signaling). Membranes were washed twice in PBST and incubated with an appropriate HRP-linked secondary antibody at 1:5000 dilution for two hours at room temperature. The membranes were washed extensively in PBST and imaged using the Femto Maximum Sensitivity Substrate (Thermo Scientific) with a FluorChem HD2 System (Cell Biosciences). Silver staining was performed on gels using a silver staining kit according to the manufacturer's instructions (Thermo Scientific).

3.2.4 Liposomal Characterization

The size and dispersion of the isolated glypican-1 and liposomes was characterized by dynamic light scattering (Malvern Zetasizer Nano ZS). Calibration was performed using 54 nm polystyrene particles. Results shown are the average of 50 measurements. To visualize the glypisomes, carbon support grids (300 mesh Cu; EM

Sciences) were treated with glow discharge at 50 mA for two minutes (Emitech K100x; Quorum Technologies). The samples were then applied to the grids and the excess liquid removed with a filter paper. The grids were then stained with a 2% uranyl acetate solution and imaged using an FEI Tecnai Transmission Electron Microscope (TEM).

3.2.5 FGF-2 Trafficking and Rab Co-localization Assays

Constructs for expressing GFP-Rab5, GFP-Rab7 and GFP-Rab11 were as described previously [125, 126]. A construct to express GFP-Rab11 was created using similar methods. For studying the effect of glypisomes on endosomal trafficking, HEK 293 cells were plated on glass bottom dishes and transfected with the constructs using lipofectamine 2000 (Life Technologies, inc.). We conjugated Alexa Fluor 594 to FGF-2 while it was bound to heparin column to prevent loss of activity as described previously [127]. Twenty-four hours after transfection, 27 ng/ml of labeled FGF-2 was added. The cells were fixed at various time points after the addition of FGF-2 and then imaged using a laser scanning confocal microscope (LSM 710; Zeiss). The percentage Rab-positive endosomes that also contained FGF-2 was quantified using 10 cells at each time point (all Rab-positive endosomes were counted for each cell). The total fluorescence intensity and nuclear intensity of FGF-2 was calculated by selecting the whole cell or DAPI-stained nucleus and measuring intensity in exposure normalized images using Photoshop software (Adobe).

3.2.6 Cell Proliferation Assay

Endothelial cells were passaged into a 96-well plate and cultured in low serum media with 2% FBS for 24 hours. Glypisomes and/or growth factors were then added to cells. After 24 hours, BrdU was added to the cells and proliferation was assessed 12 hours later using a colorimetric BrdU assay (Cell Signaling, Inc.). For the temporal bead

release study 27,000 endothelial cells/well were plated in a 12 well plate and an alginate bead containing either FGF-2 or glypisomes was added to the well, at each time point the number of cells were counted using a hemocytometer and normalized to the initial cell count.

3.2.7 Wound Healing Cell Migration Assay

Endothelial cells were grown to confluence and then cultured in low serum media (2% FBS) for 24 hours. A cell scraper was used to create a wound in each well. The glypisomes were added with FGF-2 (10 ng/ml) or VEGF-A (10 ng/ml) immediately after wounding of the monolayer. The wounds were imaged along their entire length using an inverted, phase-contrast microscope with digital camera immediately after wounding and six hours later. The average gap was calculated using Metamorph software (Molecular Devices) the rate of wound closure was calculated by taking the difference between the two measurements.

3.2.8 In Vitro Angiogenic Differentiation Assay

Multi-well culture plates were coated with growth factor depleted Matrigel (BD Biosciences) and allowed to gel for one hour at 37°C. In each well, endothelial cells were plated at a concentration of $2x10^4$ cells per well in 24-well plates. The cells were treated with the various combinations of glypisomes and growth factors. The cells were imaged after 16 hours and then quantified for average tubule length, number of tubules, and number of tubule branching points were then quantified using Metamorph software (Molecular Devices).

3.2.9 Preparation of Alginate Beads, Measurement of Release Kinetics

Equal volumes of a 4% sodium alginate solution and a 0.85% NaCl solution were mixed together. The glypisomes (1:100 dilution) and FGF (1.5 μg/100 μl) were added to

this solution. Beads were created by controlled extrusion into a 1.1% $CaCl_2$ solution through a 30G needle using a syringe pump. The beads were allowed to crosslink for 1 hour at 4°C. For measuring the release kinetics, alginate beads were put into plastic scintillation vials containing PBS with calcium and magnesium chloride. At various time points the liquid was completely removed, replaced and the harvested liquid frozen at -80C. The FGF-2 released was quantified using ELISA (R&D Systems). Glypican-1 release was quantified using an ELISA (Ray Biotech).

3.2.10 Scanning Electron Microscopy

To examine the morphology of the alginate gels, the samples were flash frozen in liquid nitrogen (-195°C) and lyophilized overnight (-110°C; 0.0005 mbar) in scintillation vials. The freeze-dried gels were sputter coated with gold discharge for 30 seconds and then loaded on the stage to image them under the scanning electron microscope (FEI Quanta 650 ESEM) with an acceleration voltage of 10 kV.

3.2.11 Statistical Analysis

All results are shown as mean ± standard error of the mean. Comparisons between only two groups were performed using a two-tailed Student's t-test. Multiple comparisons between groups were analyzed using a two-way ANOVA followed by Dunnett post-hoc testing. A two-tailed probability value of $p < 0.05$ was considered statistically significant.

3.3 RESULTS

3.3.1 Glypican-1 is Reduced in the Blood Vessels of Skin Tissues from Diabetic Patients

Our recent studies have shown that there are reduced protein levels of the syndecan family of cell surface heparan sulfate proteoglycans in diabetic mice on a high

fat diet [4]. This finding led us to hypothesize that loss of growth factor co-receptors, including glypican-1, is a key mechanism in diabetes-mediated growth factor resistance. To test if a similar phenomenon was happening in humans with diabetes, we collected skin samples from human patients and immunostained sections from these samples for glypican-1 (Figure 3.2A; Table 3.1). This analysis revealed a significant reduction in the number of glypican-1 positive cells in the arterioles from the skin of patients with type 2 diabetes (Figure 3.2B). Consistent with these findings, we performed western blotting on tissues from ob/ob and wild type mice that had been exposed to a high fat diet to 10 weeks and found a reduced amount of glypican-1 in ob/ob mice versus wild type mice with a high fat diet (Figure 3.3). Glypican-1 serves as a co-receptor for angiogenic growth factors and, consequently, its loss may lead to reduced growth factor responsiveness. To overcome this mechanism of therapeutic resistance, we created a protein therapeutic formulation of glypican-1 by embedding it in the lipid membrane of a liposome that allows it to be co-delivered with growth factors ("glypisomes"; Figure 3.4).

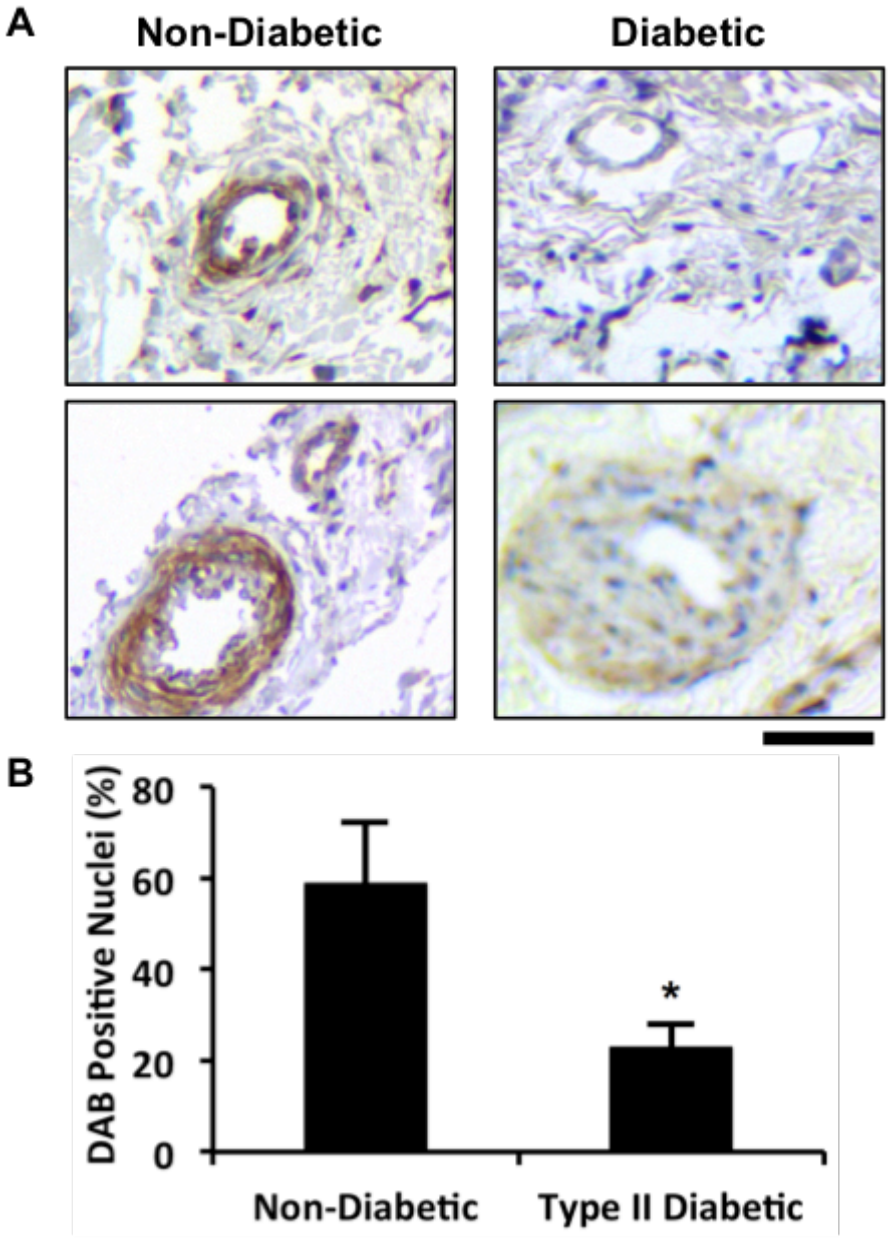

Figure 3.2 Glypican-1 is reduced in mice and human with diabetes. (A) Immunostaining for glypican-1 in skin samples from diabetic and non-diabetic patients. (B) Analysis of the number of DAB positive nuclei within arterioles within the skin. *Statistically significant different from the no growth factor and growth factor alone groups ($p < 0.05$). Bar = 50 mm.

Table 1. *Patient Sample Data*

Diabetes Status	Age	Sex
Normal	59	M
Normal	49	F
Normal	69	M
Normal	62	M
Normal	80	F
Normal	39	F
Diabetic, type II	73	M
Diabetic, type II	47	F
Diabetic, type II	80	M
Diabetic, type II	81	M
Diabetic, type II	53	M
Diabetic, type II	61	M
Diabetic, type II	67	M

Table 3.1 Table of human skin sample patient data.

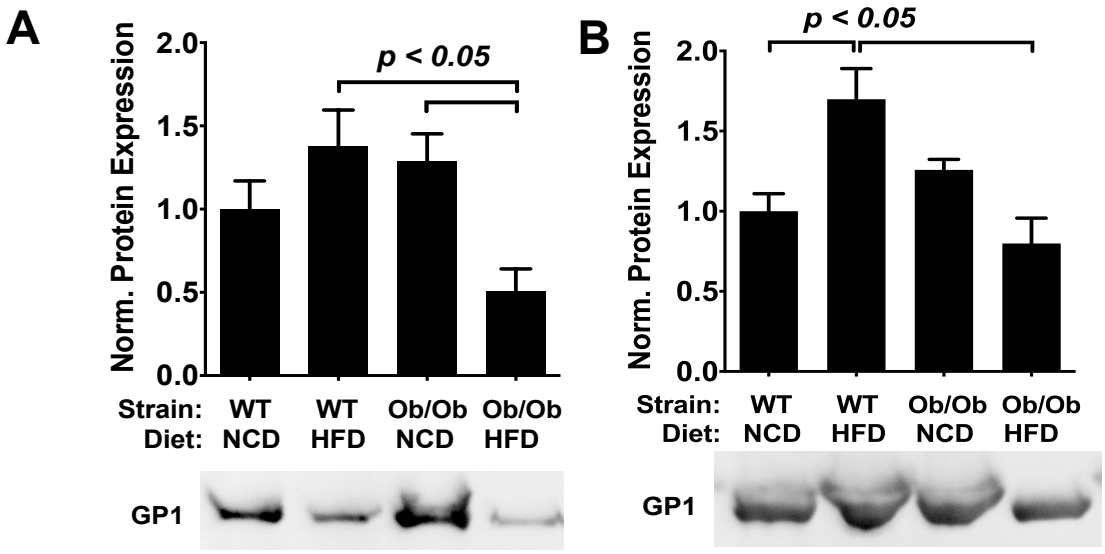

Figure 3.3 Glypican-1 is reduced in mice and human with diabetes. Wild type (WT) or ob/ob mice were given a normal or high fat diet for 10 weeks and then the tissue isolated. The tissues were sectioned and then lysed for western blot analysis. (A) Glypican-1 expression in the skeletal muscle of WT and ob/ob mice on a normal chow diet (NCD) or high fat diet (HFD). (B) Expression of glypican-1 in the cardiac muscle of WT and ob/ob mice.

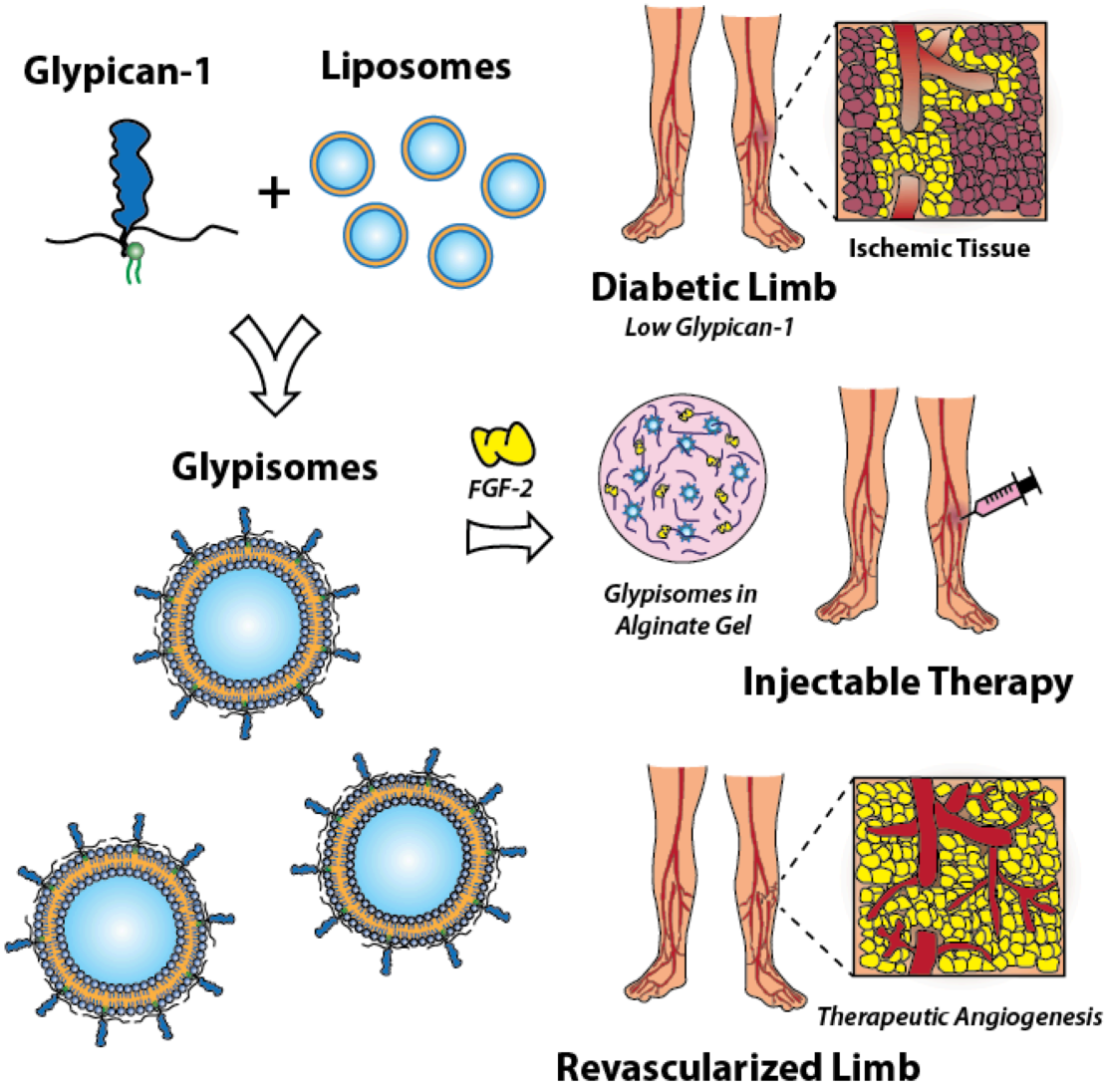

Figure 3.4 Overall diagram of glypisome-based therapeutics for the treatment of ischemia. Recombinant glypican-1 is embedded in a liposome membrane using detergent extraction. This construct is then combined with growth factors and polymerized into an alginate gel for delivery.

3.3.2 Synthesis of Glypisomes and Characterization of Release Kinetics from Alginate Carrier

To create glypisomes, we produced and isolated recombinant glypican-1 and then embedded the proteins in the membranes of 400-nm liposomes using the progressive detergent extraction method (Figure 3.5A; Figure 3.1). For delivery, the glypisomes were encapsulated in an alginate gel (Figure 3.5B). We created a set of glypisomes with varying lipid:protein ratio, ranging from 100% liposomes with no glypican-1 (L100:G0) to the isolated glypican-1 protein alone (L0:G100). We examined the size distribution of the glypisomes using transmission electron microscopy (TEM) and dynamic light scattering (DLS; Figure 3.6). As anticipated, the liposomes and glypisomes had a size distribution near to the 400-nm diameter of the liposomes following the initial extrusion step. Surprisingly, the isolated glypican-1 alone had a size of around 190 nm in diameter. This result suggests that self-association occurs for isolated glypican-1 and/or the co-isolation of lipids that creates an aggregate that has a diameter similar to that of the liposomal embedding particles. We analyzed the isolated glypican-1 for cholesterol to see if there was association of lipid rafts with the glypican-1 and this analysis revealed undetectable levels of cholesterol (data not shown). We encapsulated the mid-range composition glypisomes (L20:G80) in an alginate gel combined with FGF-2 and measured the release of FGF-2 over time using ELISA. The incorporation of glypisomes led to a similar response compared to FGF-2 alone, with a slight increase in the release of FGF-2 for the later time points out to 14 days (Figure 3.7A). The release profile for glypican-1 from these beads was slower than that of FGF-2, likely reflecting the larger size of the proteoliposome (Figure 3.7B).

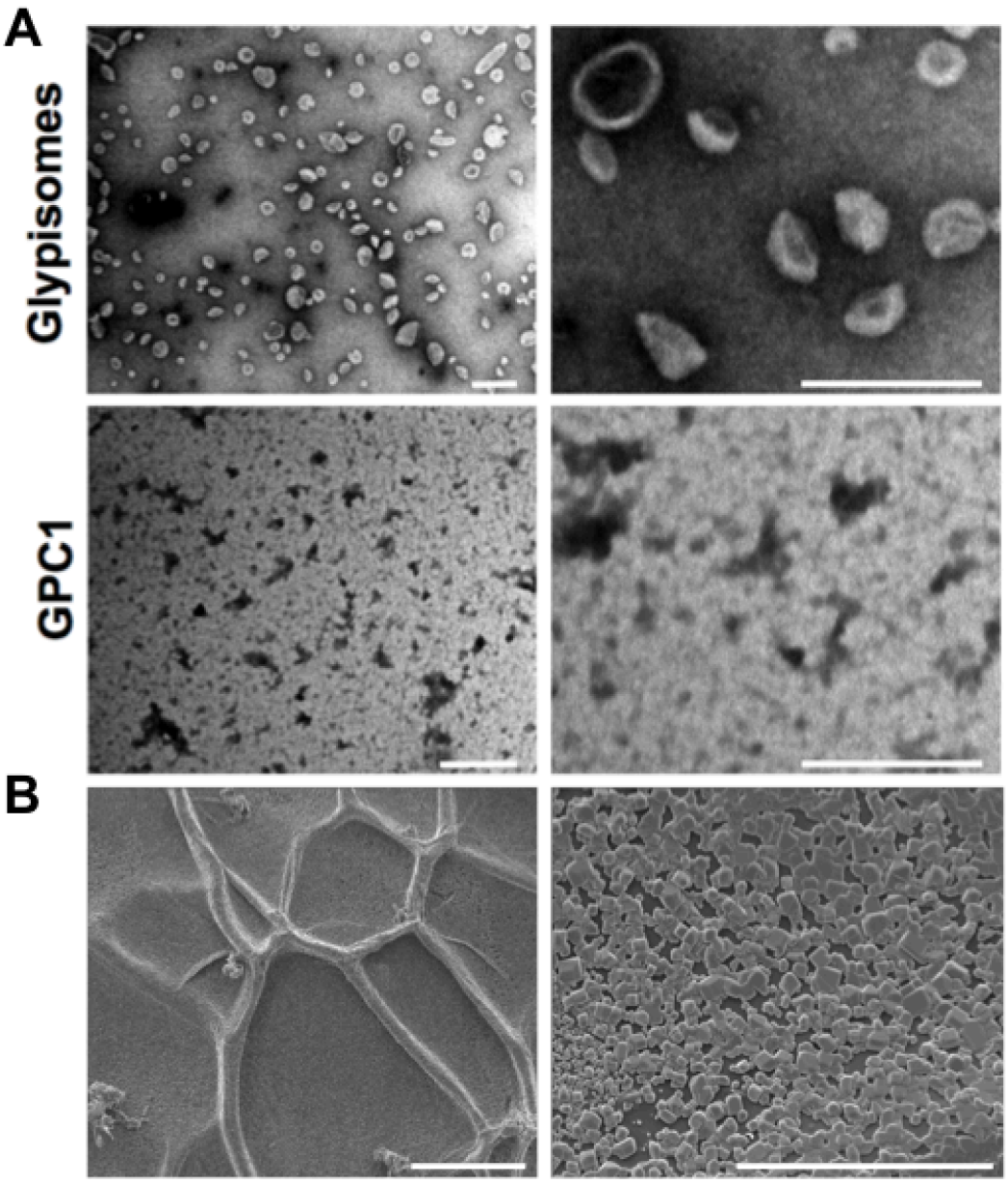

Figure 3.5 Characterization of glypisomes and alginate delivery gel. (A) Visualization of glypisomes and isolated glypican-1 (GPC1) by transmission electron microscopy. Bar = 200 nm. (B) Scanning electron microscopy visualization of the alginate gel after polymerization. Left bar = 100 μm and right bar = 30 μm.

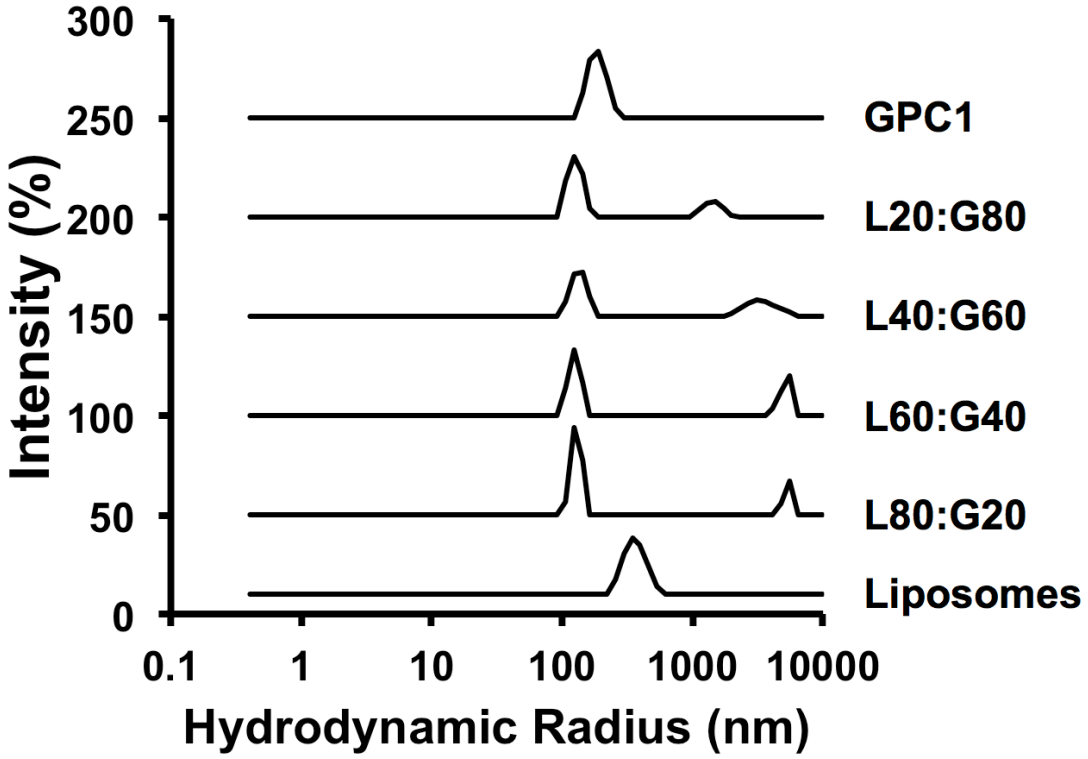

Figure 3.6 Dynamic light scattering of isolated glypican-1 (GPC1), liposomes and glypisomes. Glypisomes are listed as composition ratio between lipid (L%) and protein (P%).

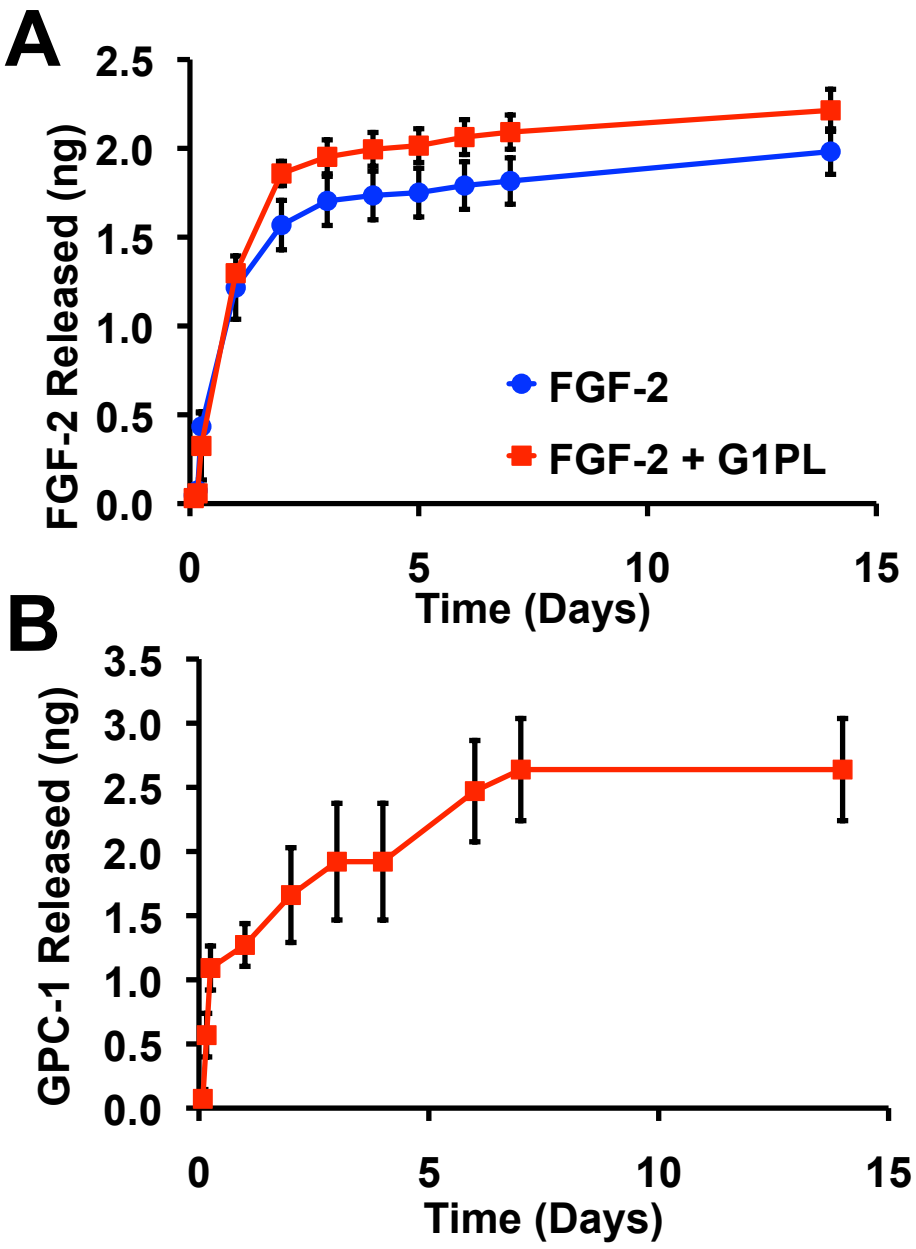

Figure 3.7 (A) Release kinetics of FGF-2 from alginate gel with and without glypisomes added. (B) Release kinetics of glypican-1 from alginate gels containing FGF-2 and glypisomes.

3.3.3 Glypisomes Enhance Proliferation and Migration of Endothelial Cells Stimulated with FGF-2 or VEGF

To optimize the effects of glypisome composition on activity, we measured the ability of glypisomes to enhance FGF-2 or VEGF activity in a proliferation assay. For FGF-2, glypisomes with higher levels of glypican-1 enhanced FGF-2 induced proliferation of endothelial cells by nearly three-fold (Figure 3.8). Notably, the liposome alone had an enhancing effect on proliferation in comparison to FGF-2, an effect we had observed in previous studies using a similar liposome formulation [127]. Interestingly, isolated glypican-1 also increased proliferation but not to the full extent as the mid-range composition glypisomes. In contrast, VEGF-induced proliferation was not significantly altered by the addition of liposome or glypisomes (Figure 3.8). The isolated glypican-1 reduced the proliferation in response to VEGF to baseline levels. Glypisomes and isolated glypican-1 had only small effects on endothelial proliferation in the absence of growth factors (Figure 3.9). Human endothelial cells treated with glypisomes or FGF-2 with glypisomes encapsulated within an alginate bead show a significant increase in proliferation after one day and FGF-2 and glypisomes show a significant increase in proliferation over alginate alone and glypisomes after the second day (Figure 3.10). In addition, we examined the effect of the glypisomes on the migration of endothelial cells in an *in vitro* scratch wound assay. We found that for the L20:G80 composition of glypisomes there was no enhancement of FGF-2 induced migration but VEGF induced migration was increased (Figure 3.11). We found that glypisomes in the absence of growth factors have no significant effect on endothelial migration (Figure 3.12).

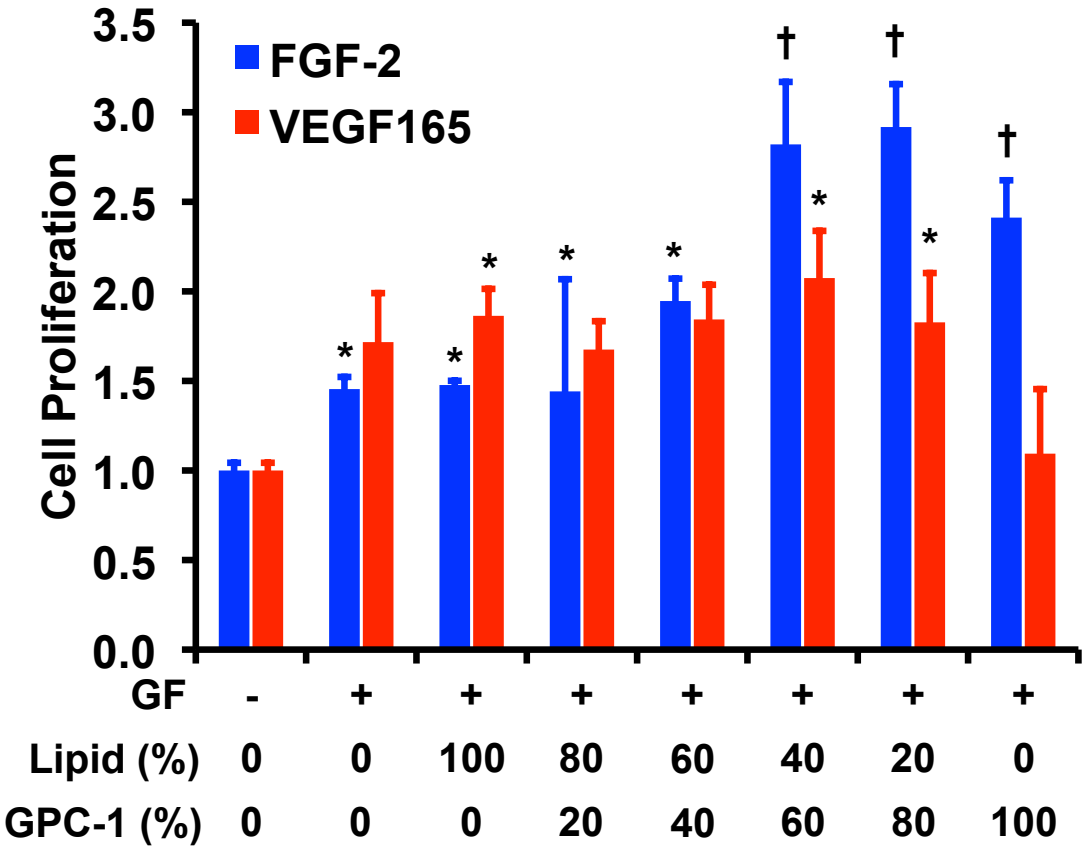

Figure 3.8 Proliferation of human endothelial cells after stimulation with growth factors and glypisomes with varying ratios between lipid and recombinant glypcian-1 (GPC-1). Endothelial cells were treated with 10 ng/ml FGF-2 or 10 ng/ml $VEGF_{165}$ and glypisomes, and then proliferation was measured from BrdU incorporation assay. *Statistically significant difference between group and the no growth factor group ($p < 0.05$). †Statistically significant different from the no growth factor and growth factor alone groups ($p < 0.05$).

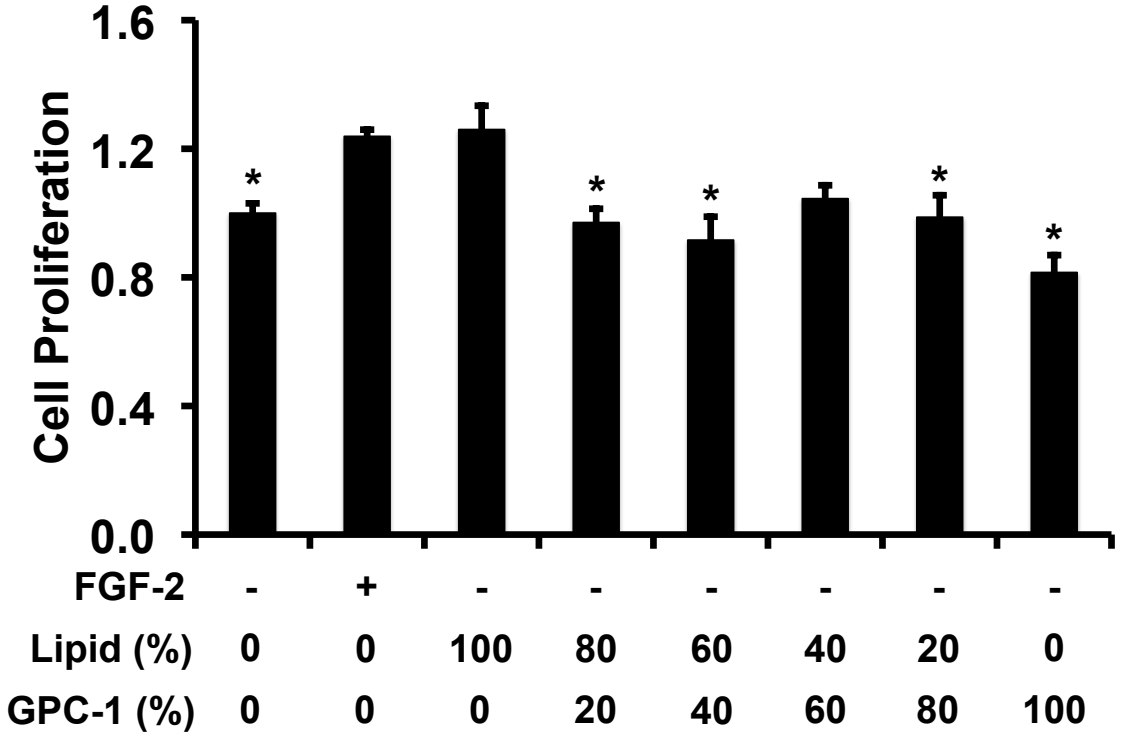

Figure 3.9 Effect of glypican-1 and glypisomes on endothelial cell proliferation in the absence of growth factors. Proliferation of human endothelial cells after stimulation with varying ratios between lipid and recombinant glypican-1 (GPC-1). Cells were treated with the glypisomes, FGF-2 (10 ng/ml) treatment was used as a positive control. *Statistically significant difference from FGF-2 treated cells and cells treated with liposomes without glypican-1 ($p < 0.05$).

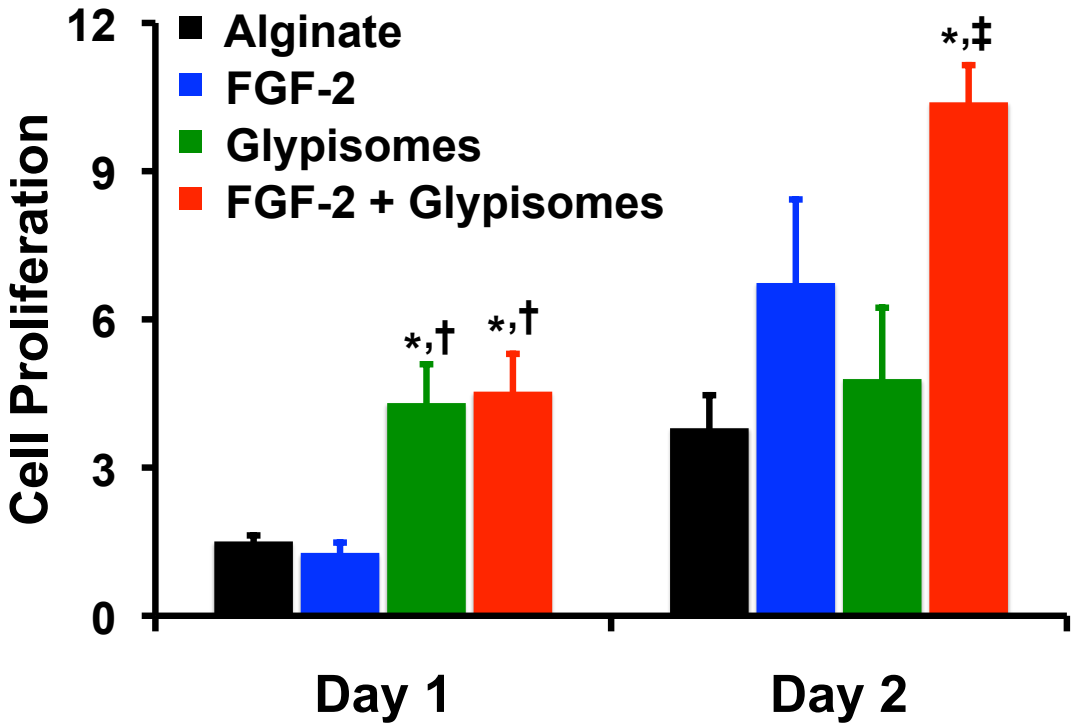

Figure 3.10 Human endothelial cells were treated with an alginate bead containing FGF-2 and/or glypisomes. The number of cells/well was counted at each time point. *Statistically significant difference between group and alginate alone ($p < 0.05$). †Statistically significant difference between group and FGF-2 ($p < 0.05$). ‡Statistically significant difference between group and glypisomes ($p < 0.05$).

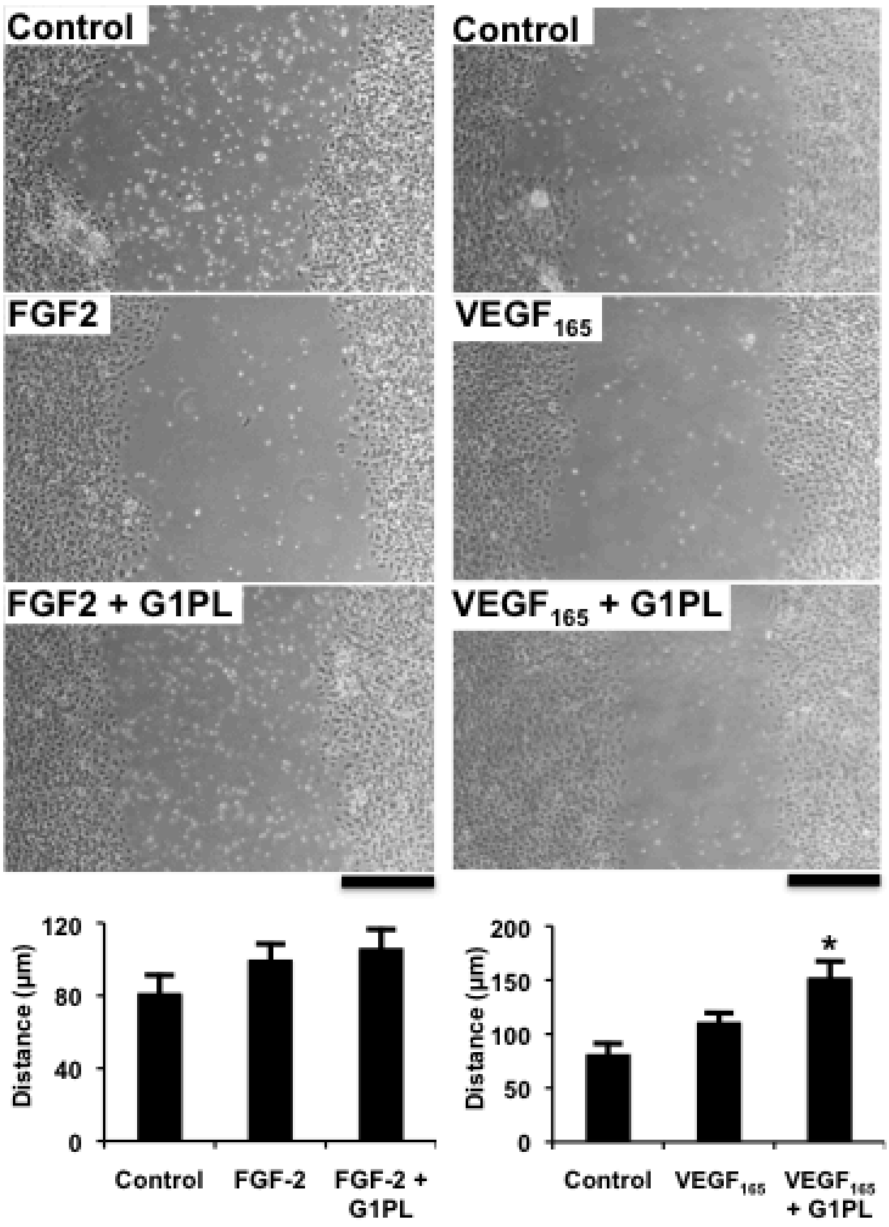

Figure 3.11 Wound closure after scratch wounding of an endothelial monolayer. Cells were treated with 10 ng/ml FGF-2 or 10 ng/ml VEGF at the time of injury. The glypisomes (G1PL) with a lipid to glypican-1 protein ratio of 20:80 were used. *Statistically significant different from the no growth factor and growth factor alone groups ($p < 0.05$). Bar = 200 μm.

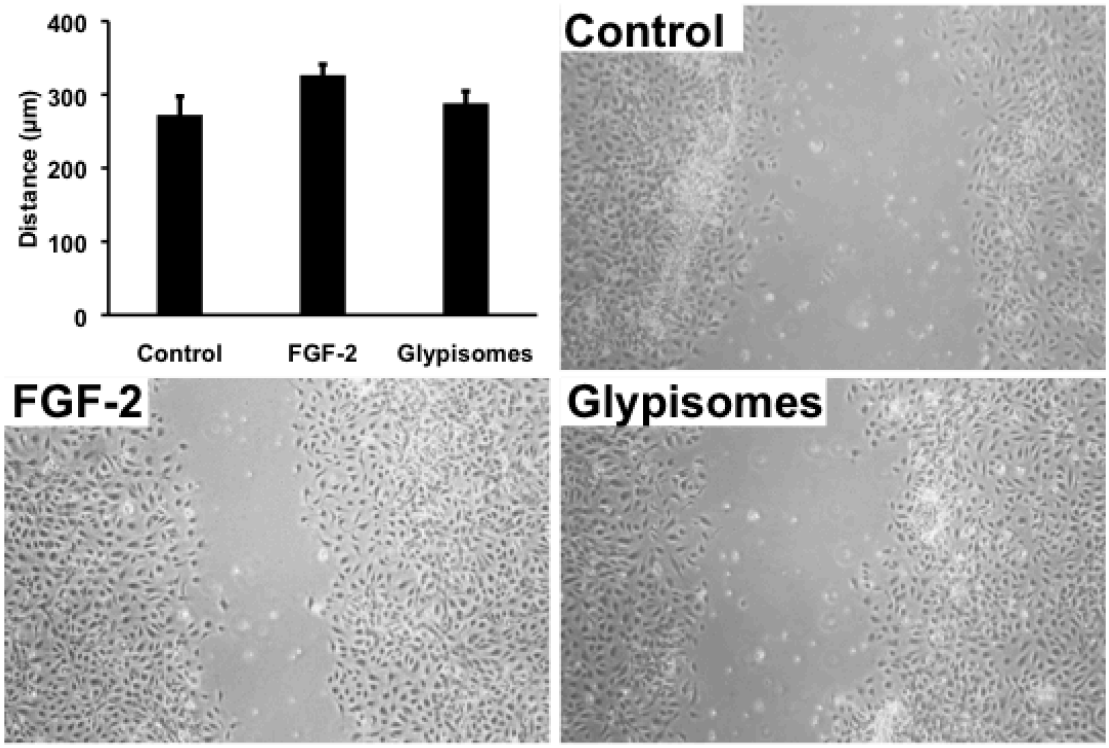

Figure 3.12 Effect of glypisomes on endothelial cell migration in the absence of growth factor. Wound closure after scratch wounding a human endothelial monolayer. Cells were treated with either glypisomes or FGF-2 (10 ng/ml) at the time of injury. Glypisomes with a lipid to glypican-1 protein ratio of 20:80 were used in the studies.

3.3.4 Glypisomes Enhance FGF-2 Induced In-Vitro Tube Formation

We next examined the effect of glypisome treatment on growth factor activity in an *in vitro* tube formation assay of angiogenic differentiation. Endothelial cells were grown on growth factor depleted matrigel, serum starved and then treated with growth factors or growth factors in combination with the glypisomes of varying composition. For glypisomes in combination with FGF-2 there was a marked increase in branch points, tube length and number of tubes formed in cells treated with the glypisomes with higher levels of glypican-1 (Figure 3.13). When glypisomes were delivered with VEGF we found they enhanced tube length with midrange concentrations of glypican-1 (60:40 and 40:60 lipid to protein ratio; Figure 3.14). Isolated glypican-1 also increased VEGF-induced differentiation but to a lesser extent than the midrange composition glypisomes. There were no significant alterations in the number of branch points and number of tubes with glypisome treatment in combination with VEGF.

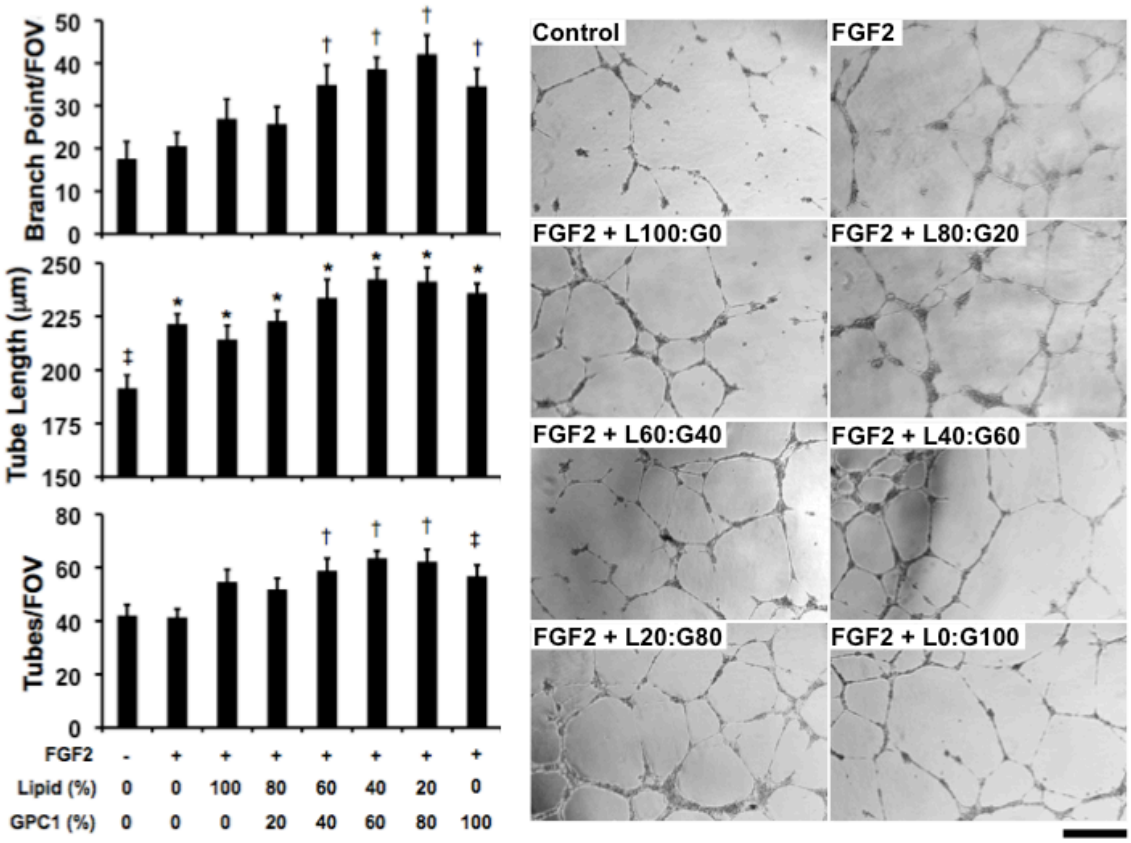

Figure 3.13 Glypisomes enhance FGF-2 induced endothelial tube formation. Endothelial cells grown on growth factor reduced matrigel and the cells were treated with glypisomes with varying composition and 10 ng/ml FGF-2. After 16 hours the formation of tubes was assessed by phase contrast microscopy. *Statistically significant difference between group and the no growth factor group ($p < 0.05$). †Statistically significant difference from the no growth factor and growth factor alone groups ($p < 0.05$). Bar = 200 μm.

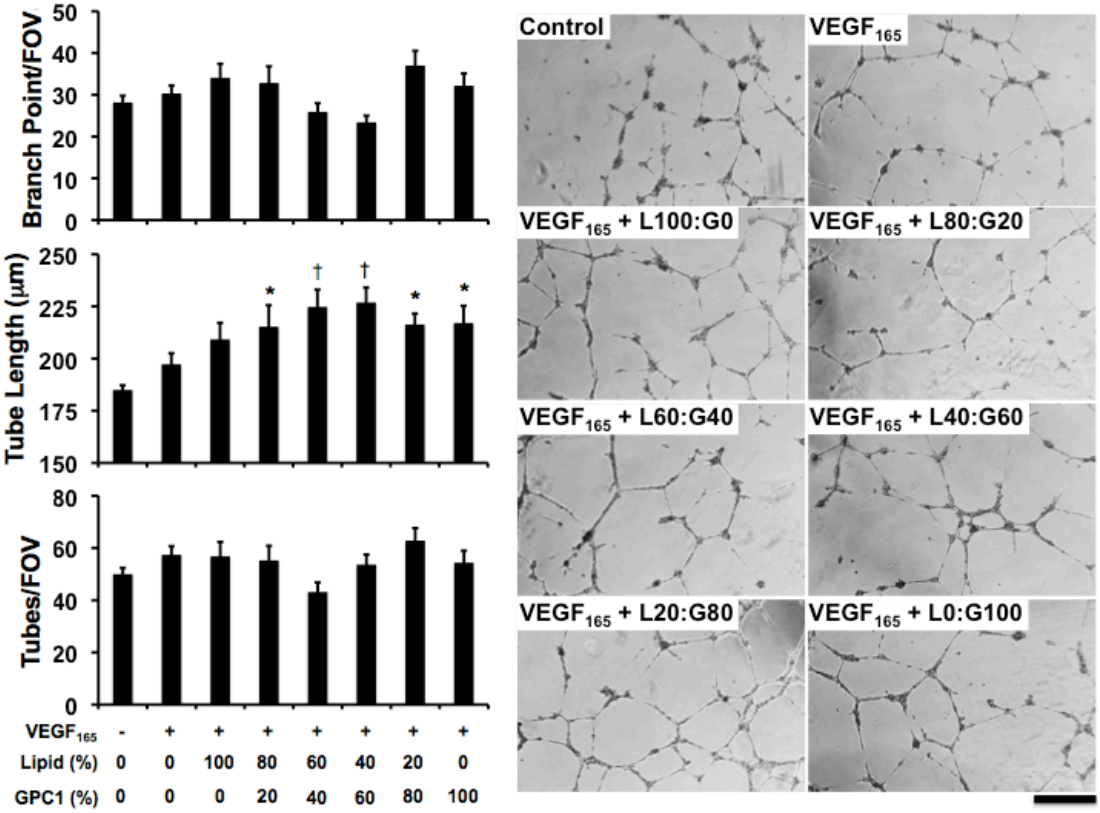

Figure 3.14 Glypisomes enhance VEGF induced endothelial tube formation. Endothelial cells grown on growth factor reduced matrigel and the cells were treated with glypisomes with varying composition and 10 ng/ml VEGF. After 16 hours the formation of tubes was assessed by phase contrast microscopy. *Statistically significant difference between group and the no growth factor group ($p < 0.05$). †Statistically significant different from the no growth factor and growth factor alone groups ($p < 0.05$). Bar = 200 μm.

3.3.5 Glypisomes Increase FGF-2 Induced Signaling Through the AKT Pathway and Enhance Both FGF-2 Uptake and Endosomal Trafficking

We examined whether glypican-1 proteoliposomes could increase the downstream signaling pathways induced by FGF-2. For the ERK1/2 pathway, we found that the cell signaling response with and without glypisomes was very similar (Figure 3.15A). However, for the AKT pathway there was a moderate increase in the AKT signaling after 60 minutes (Figure 3.15B). Notably, neither pathway was appreciably activated by the glypisomes without the growth factor. To further explore the mechanism of glypisome enhancement of FGF-2 activity, we treated cells expressing GFP-labeled versions of the endosomal trafficking proteins Rab5, Rab7 or Rab11 with flurophore-labeled FGF-2 (Figure 3.16). These proteins are markers of early (Rab5) and late (Rab7) endosomes as well as endosomal recycling (Rab11). We found increased numbers of Rab5 and Rab7 positive endosomes that contained FGF-2 at all time points after 15 or 30 minutes, respectively, with treatment with glypisomes. At 120 minutes, there were increased numbers of Rab11 positive endosomes that contained FGF-2 in the glypisome treated group. We also measured the total cell fluorescence intensity for FGF-2 and found this was moderately increased for the glypisome group (Figure 3.17A). Finally, we measured the intensity of FGF-2 in the nucleus for the cells and found increased nuclear localization at the 15-minute time point for the glypisome group (Figure 3.17B). Together, these results support the notion that glypisomes have activity through a moderate enhancing effect on AKT signaling, by increasing FGF-2 uptake and through enhancing endosomal processing of FGF-2.

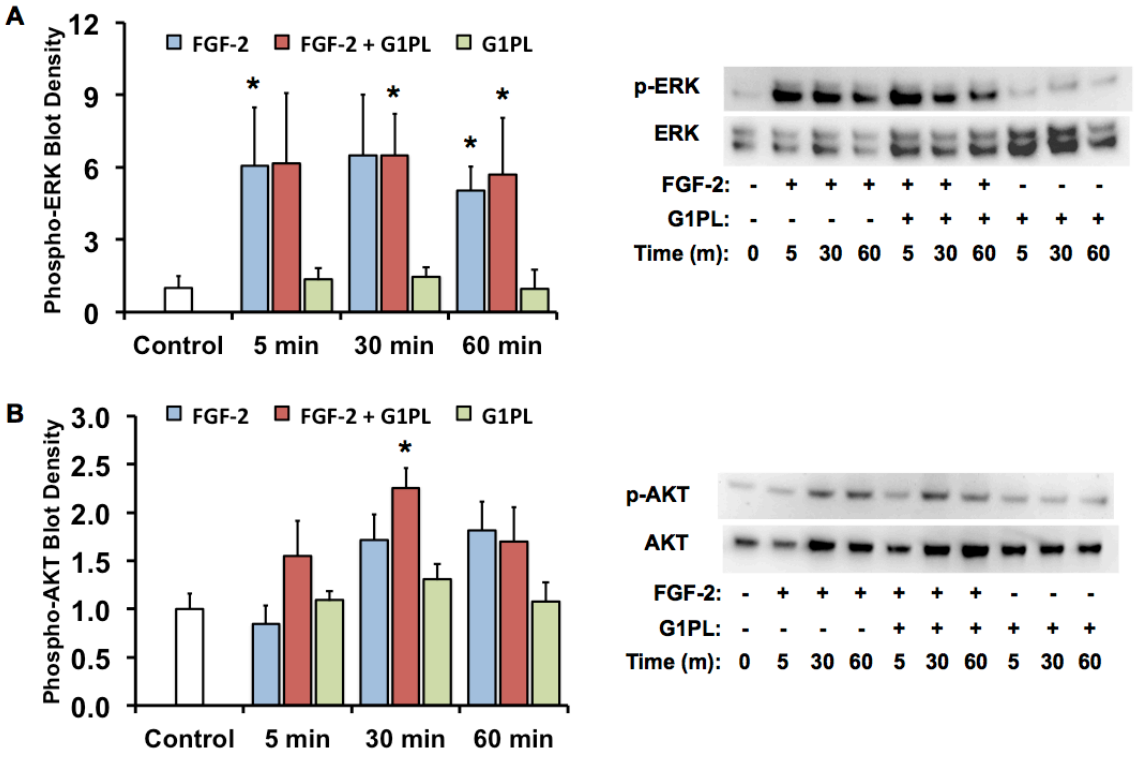

Figure 3.15 Effect of glypisomes on the intracellular signaling response to FGF-2. Endothelial cells were serum starved and stimulated with 10 ng/ml FGF-2 and/or glypisomes. The cells were lysed at various time points and western blotted for signaling mediators. *Statistically significant difference between group and control group ($p < 0.05$).

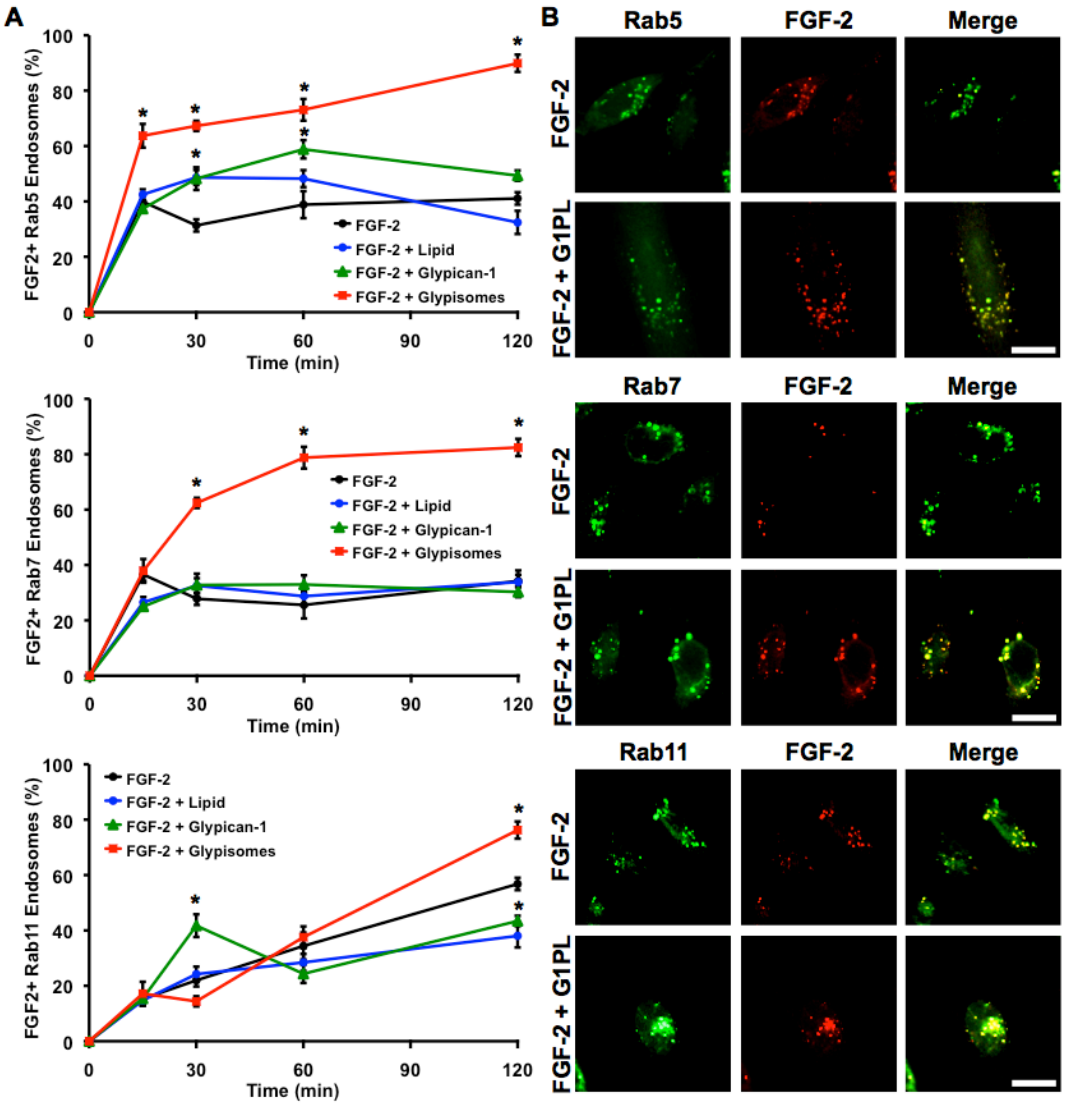

Figure 3.16 Glypisomes increase endosomal trafficking of FGF-2 in HEK-293 cells. (A) Quantification of the percentage of Rab5, Rab7 or Rab11 positive (GFP) endosomes that contained FGF-2 after treatment of HEK-293 cells with various conditions. (B) Representative confocal images of cells treated with FGF-2 or FGF-2 with glypisomes at 120 min post treatment. *Statistically significant difference between the group and the FGF-2 only group at the same time point ($p < 0.05$). Bar = 20 μm.

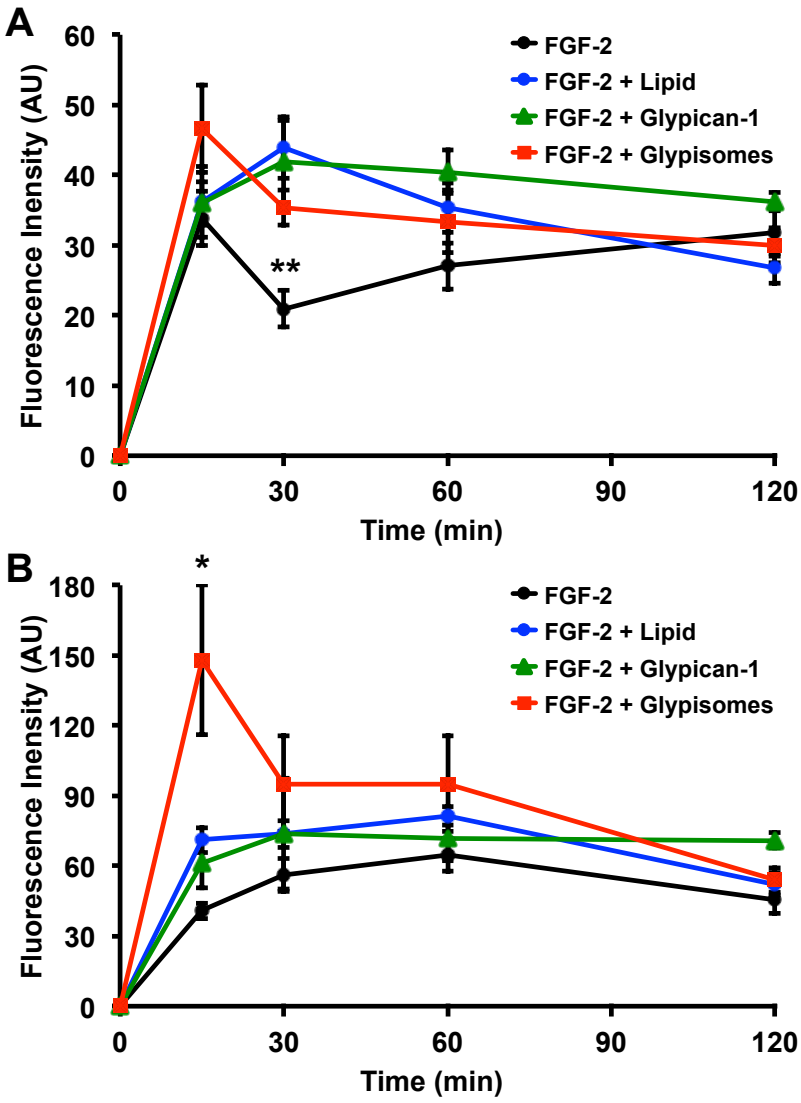

Figure 3.17 Glypisomes increase total and early nuclear concentration of FGF-2 in cells. HEK-293 cells were treated with fluorescently labeled FGF-2 and fixed at various time points. The cells were stained with DAPI to define the nuclear area. The fluorescence was measured for (A) the total cell and (B) nuclear area from confocal images. *Statistically significant difference compared to the FGF-2 alone group ($p < 0.05$). **Statistically significant difference with FGF-2 with glypisomes group ($p < 0.05$). Bar = 20 μm.

3.4 DISCUSSION

It has been challenging to develop angiogenic therapies that are effective in the context of human patients with disease. Here, we have shown that glypican-1 is reduced in the skin from patients with type 2 diabetes using immunohistochemical staining. This type of analysis is semi-quantitative but the large difference between the patient groups would support that there may be significant alterations in glypican-1 levels in diabetic patients. The implication of these findings is that loss of glypican-1 may contribute to reduced tissue sensitivity to growth factors and inhibit the response to angiogenic therapies. Consequently, in spite of high levels of growth factor delivered in a therapeutic context the tissue would likely not be able to mount an effective angiogenic response. Our approach to overcoming this potential mechanism of growth factor resistance was to create a therapy that can deliver the missing glypican-1 to the tissues without the need for gene therapies or the addition of exogenous cells. We delivered the glypican-1 embedded in a liposome membrane to simulate the endogenous glypican-1 presentation and to facilitate uptake of the protein. The formulation is stable when encapsulated in alginate and can be delivered locally to ischemic areas of the body. We show here that this method is very effective in increasing FGF-2 activity *in vitro*.

Our *in vitro* studies revealed that glypisomes enhance growth factor activity but there were significant differences in the enhancement response between FGF-2 and VEGF. The proliferative response to FGF-2 was increased more by glypisomes than those stimulated with VEGF. In contrast, the cells simulated with VEGF and glypisomes had enhanced migration where FGF-2 simulated cells did not. Also, in the *in vitro* tube formation assay there were marked differences between VEGF and FGF stimulated network formation in the presence of glypisomes. The primary effect for cells treated with FGF-2 was an increase in branch points with a moderate increase in tube length. However, for cells treated with VEGF the effect was primarily an increase in the length of the tubes without little or negative effects on the branch points. Glypican-1 has been shown to increase the activity of both these growth factors [41, 44, 48] and the diversity of the response to glypisome may reflect the differences in the vascular networks that are induced FGF-2

and VEGF. For instance, FGF-2 is known to induce a strong proliferative response where as VEGF has been associated with a stronger effect on phenotypic differentiation [128]. This is reflected in the vascular networks that form when delivered exogenously with FGF-induced networks having fewer branch points and lower permeability than VEGF-induced networks [128].

Our studies demonstrated markedly increased uptake and concentration of FGF-2 in early (Rab5+) and late (Rab7+) endosomes. Prior studies have shown that glypican-1 serves as a low affinity receptor for FGF-2 that stabilizes the FGF-2/FGF receptor complex.[44] In addition, heparan sulfate proteoglycans can serve as mediators of cellular uptake of FGF-2 [129]. Endogenous cell surface glypican-1 is known to be constitutively endocytosed in many cell types where its heparan sulfate chains are partially degraded and the protein is transported to the Golgi for glycosylation [130]. Upon activation, the FGF-2/FGFR1 complex is internalized through a clathrin-mediated mechanism and, subsequently, FGFR1 is found localized with first early endosomes and then late endosomes [131]. Our findings of increased FGF-2 signaling, uptake and endosomal processing are consistent with the hypothesis that exogenous glypican-1 acts similarly to endogenous glypican-1 in stabilizing the receptor-growth factor complex. However, we observed increased activity of the liposome-embedded glypican-1 in comparison to free glypican-1. The liposomal component may facilitate the uptake of a larger complex of FGF-2/FGFR-1 bound to a glypisome associated with additional FGF-2 not bound to the receptor complex on the other parts of the liposome. This concept would explain how the glypisome could increase uptake significantly while having only moderate effects on the initial FGF-2 signaling.

3.5 CONCULSIONS

Here we have shown that we are able to purify recombinant glypican-1, and insert into a liposomal carrier to create the glypisomes. We have optimized the concentration of glypican-1 that leads to the greatest angiogenic response in several *in vitro* assays. The

glypisomes tend to yield the greatest angiogenic response when co-delivered with FGF-2, and while we see some response with when co-delivered with VEGF, it is significantly muted in all of our *in vitro* assays except for endothelial migration. We have also elucidated several possible mechanisms by which co-delivery of glypisomes with FGF-2 may induce a pro-angiogenic response.

Chapter 4: In Vivo Validation of Glypisomes

4.1 Introduction

In the previous chapter we optimized the concentration concentrations of glypican-1 required to maximize angiogenic response *in vitro* and characterized a potential mechanism for this response. Now, using these glypisomes, we plan to examine their affectivity at overcoming disease associated growth factor resistance by testing them in a mouse hind limb ischemia model. For our *in vivo* models we plan to only look at co-delivery with FGF-2, as we saw muted effects when co-delivered with $VEGF_{165}$ *in vitro*. Initially we will look at the effect in healthy C57Bl/6 mice (WT) and then in the disease model, ob/ob mice. Previous work has shown that these ob/ob mice do in fact exhibit disease mediated growth factor resistance[4]. These *in vivo* experiments will give us a better idea of the efficacy of our glypisome and growth factor treatment. We plan to look at our treatments ability to induce angiogenesis initially in a wild type or healthy model of hind limb ischemia. Once we have shown that we are able to induce neovascularization in healthy animals we plan to move disease model, to see if we can overcome disease-induced growth factor resistance.

4.2 Materials and Methods

4.2.1 Animal Studies

Studies involving animals were performed with the approval of the University of Texas at Austin Institutional Animal Care and Use Committee (IACUC) and in accordance with NIH guidelines for animal care. Wild-type mice or ob/ob mice (B6.Cg-Lep^{ob}/J; Jackson Labs) were used in the studies. The ob/ob mice were fed a high fat diet (Research Diets D12331) for 10 weeks prior to performing experiments. To perform the

hind limb ischemia studies, mice were anesthetized with 2% isofluorane gas and a longitudinal incision was made in the inguinal region of the right thigh. The femoral artery was separated from the femoral vein and nerve, and then double ligated with 6-0 silk sutures at two locations and the artery severed at each ligation. Alginate beads containing the various treatments were then implanted in the pocket created by the surgery. A total of 3 μg FGF-2 and 19.5 ng of glypican-1 was implanted per mouse. The incision was then closed with vicryl sutures. Relative blood flow between the ischemic and contralateral control limb was measured at days 1, 3, 5, 7, and 14 using laser speckle imaging as described previously [132]. At day 14, the mice were sacrificed and the thigh/calf muscles were harvested and frozen in liquid N_2 cooled isopentane.

4.2.2 Immunostaining and Histological Analysis

In the hind limb ischemia mouse model, the thigh and calf muscles were harvested from the mice, fixed in formalin and then processed for paraffin sectioning. The mouse tissue samples were cut with a microtome to obtain 6 μm thick sections were then stained using hematoxylin and eosin for overall morphology. The sections were deparaffinized and treated for 3 hours with antigen retrieval solution (Dako) at 80°C. The sections were cooled to room temperature and blocked with 20% fetal bovine serum for 45 minutes and then immunostained with the Envision+ Dual Link Kit (Dako) using a 1:100 dilution of primary antibody to PECAM-1 (Millipore) or a 1:500 dilution of primary antibody to glypican-1 (Thermo Scientific). Following staining, the samples were imaged using a bright field microscope (Meiji). For fluorescent immunostaining, the sections were treated as described as above but then treated with primary antibodies to glypican-1, PECAM-1 or a-SMA (Abcam). The staining was detected after treatment with fluorescently labeled secondary antibodies. The number of fibers with ischemic

changes was quantified in a minimum of three low magnification images per mouse. Histological sections from the contralateral limb were used as a control for artifacts of histological processing. Following immunostaining for PECAM-1, the number of small and large vessels was counted in images from each of the mice.

4.2.3 Statistical Analysis

All results are shown as mean ± standard error of the mean. Comparisons between only two groups were performed using a two-tailed Student's t-test. Multiple comparisons between groups were analyzed using a two-way ANOVA followed by Dunnett post-hoc testing. A two-tailed probability value of $p < 0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Local Delivery of Glypisomes Enhances FGF-2 Induced Revascularization in the Ischemic Hind Limb of Wild Type Mice

Both exogenous FGF-2 and VEGF have been shown to enhance revascularization in animal models of ischemia [133, 134]. Based on our *in vitro* studies, glypisomes appeared to be more effective in enhancing FGF-2 activity in comparison to VEGF. We induced hind limb ischemia in mice by ligating the femoral artery and implanted alginate beads that encapsulated FGF-2 or FGF-2 with the glypisomes (L20:P80; Figure 4.1). Perfusion in the ischemic limb and contralateral control limb was monitored using laser speckle imaging over 14 days. We found that glypisomes enhanced FGF-2 activity and lead to nearly twice the relative perfusion in the ischemic limb of glypisome treated mice after 14 days in comparison to those treated with FGF-2 alone (Figure 4.2A and Figure 4.2B). Following the induction of ischemia, the muscle fibers can undergo ischemic changes that include the loss of fibers within the muscle. We quantified the number of

fibers with ischemic changes that appeared as holes on histological analysis (Figure 4.3A and Figure 4.3B). Care was taken to differentiate the ischemic changes from artifacts of histological processing by the sections to those from the non-ischemic control limb for each animal. This analysis demonstrated a marked reduction in the ischemic changes in the glypisome treated mice. Immunohistochemical staining for endothelial cells (PECAM-1) and subsequent analysis revealed increased capillary density in the thigh and calf muscles, consistent with the increased perfusion observed by laser speckle imaging (Figure 4.4A and Figure 4.4B). An analysis of larger vessels (diameter > 25 μm) revealed increased arteriogenesis in the thigh muscle of glypisome treated mice (Figure 4.4C).

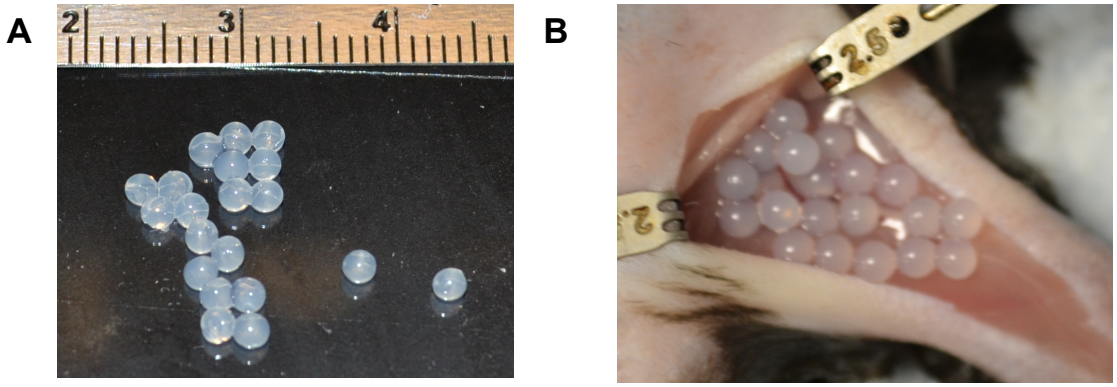

Figure 4.1 Glypisomes enhance therapeutic angiogenesis with FGF-2 in hind limb ischemia. (A) Optimal composition glypisomes were encapsulated in alginate beads. (B) Beads implanted at the site of injury at the time of surgery.

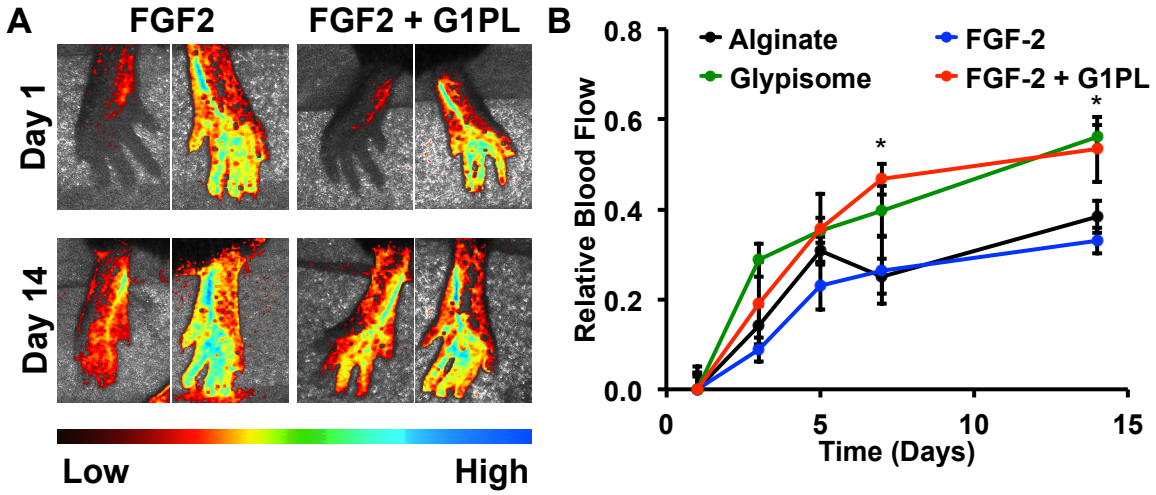

Figure 4.2 (A) Laser speckle contrast imaging was used to assess blood perfusion in the feet of the mice over time. Mice were given either alginate beads with FGF-2 or FGF-2 with glypisomes (G1PL). (B) Quantitative analysis of the perfusion of the feet after induction of hind limb ischemia. Relative blood flow was defined as the speckle contrast ratio between the ischemic limb and the control limb. *Statistically significant difference between the G1PL and G1PL + FGF-2 groups versus FGF-2 alone group at the same time point ($p < 0.05$).

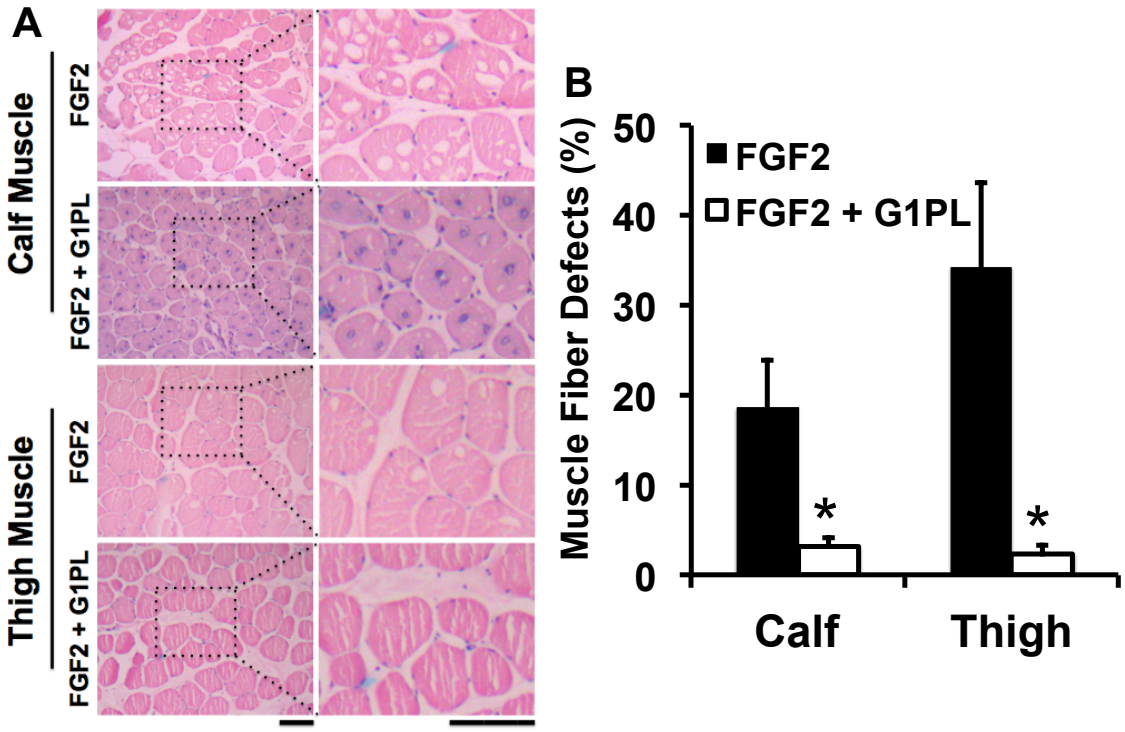

Figure 4.3 (A) Histological analysis of the calf and thigh muscles of the ischemic limb after 14 days of treatment with FGF-2 or FGF-2 with glypisomes (G1PLs). (B) Ischemic changes in the muscle fibers were reduced in the calf muscle with FGF-2 and glypisome treatment in comparison to FGF-2 alone. Ischemic changes included the loss of muscle fibers/altered morphology with the tissue. *Statistically significant difference with FGF-2 alone group ($p < 0.05$). Bar = 100 μm.

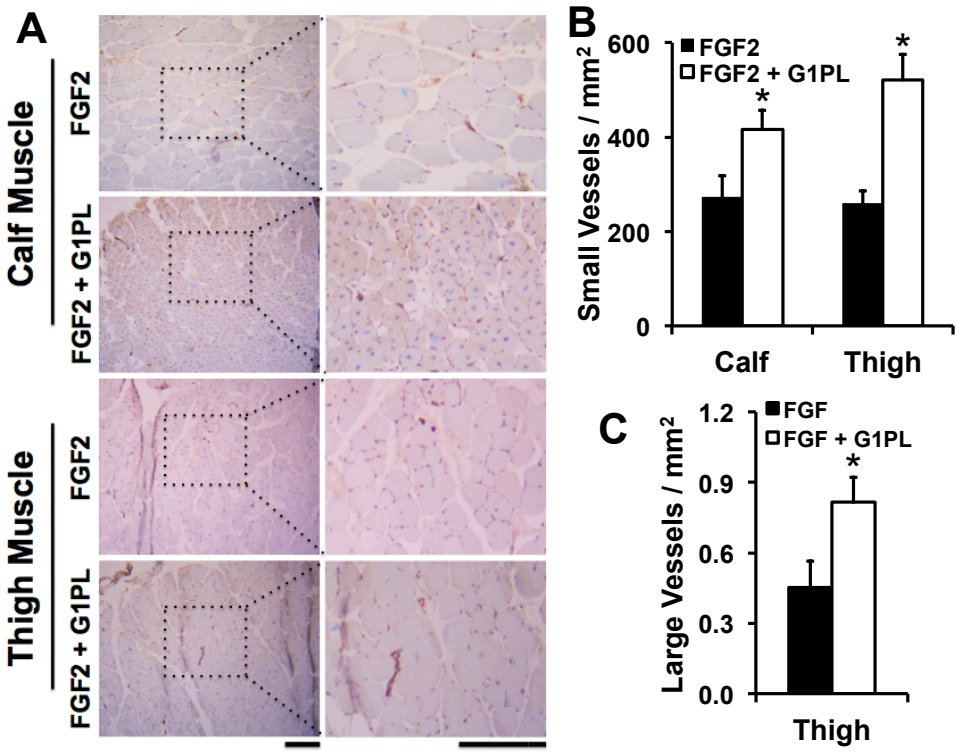

Figure 4.4 (A) Immunohistochemical staining for blood vessels (PECAM-1) in the thigh and calf muscles. (B) Quantification of small vessel density. (C) Quantification of large vessel density (diameter > 25 mm). *Statistically significant difference with FGF-2 alone group ($p < 0.05$). Bar = 100 μm.

4.3.2 Local Delivery of Glypisomes Enhances FGF-2 induced Revascularization in the Ischemic Hind Limb of Diabetic Mice

We repeated a similar study in ob/ob mice that had been treated with a high fat diet for 10 weeks. These animals have severe diabetes and obesity, and our group has shown them to be resistant to FGF-2 stimulation [4]. In these animals, we found that glypisomes also improved perfusion recovery after femoral ligation (Figure 4.5). We did not see any significant differences in morphology of the muscle (Figure 4.6) however, our speckle data was confirmed by an increase in vascularization (Figure 4.7). Consistent with these findings, we found that there were less signs of ischemia in the toes of the animals treated with glypisomes (Figure 4.8). To examine if glypican-1 delivery led to changes in glypican-1 in the tissues of the mice treated with glypisomes, we immunostained sections from the ischemic limbs of the mice for PECAM-1 and glypican-1. There was qualitatively higher diffuse glypican-1 staining within the muscles of the mice that had glypican-1 delivery (Figure 4.9). We further confirmed increased numbers of arterioles in the muscles of the mice by performing co-immunostaining for PECAM-1 and α-SMA (Figure 4.10).

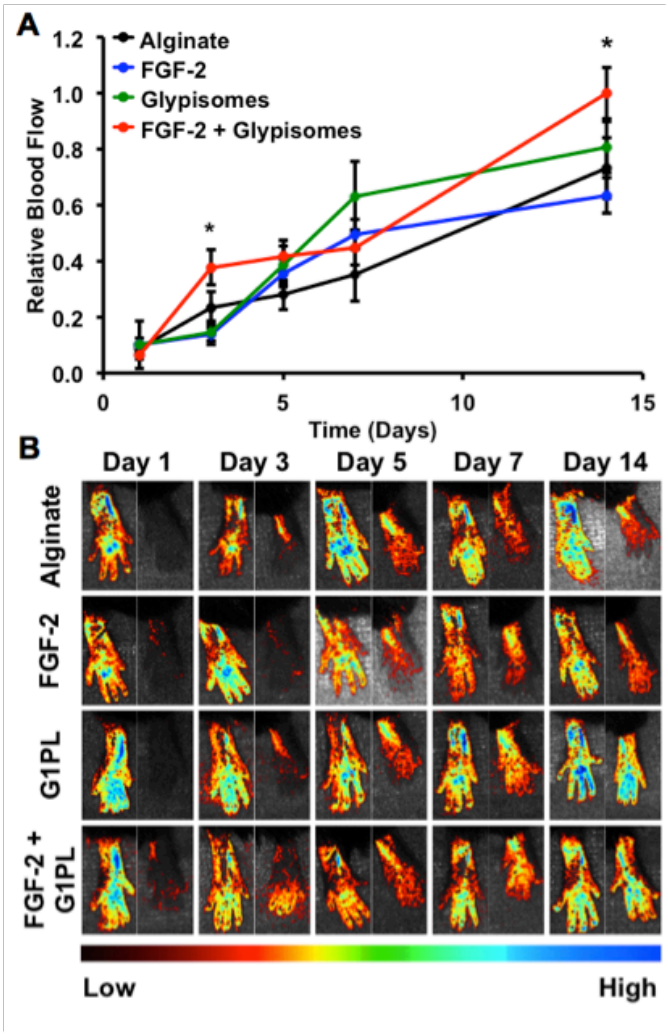

Figure 4.5 Glypisomes enhance therapeutic angiogenesis when delivered with FGF-2 in an ob/ob hind limb ischemia model. (A) Quantitative analysis of laser speckle contrast imaging gives relative blood flow analysis of perfusion measured over time. Relative flow defined as perfusion in the ischemic limb over the perfusion in the contralateral limb. (B) Representative laser speckle contrast images of each treatment group at 1, 3, 5, 7, and 14 days post injury. Right paw is from ischemic limb and left is the contralateral control. *Statistically significant difference with FGF-2 alone group ($p < 0.05$).

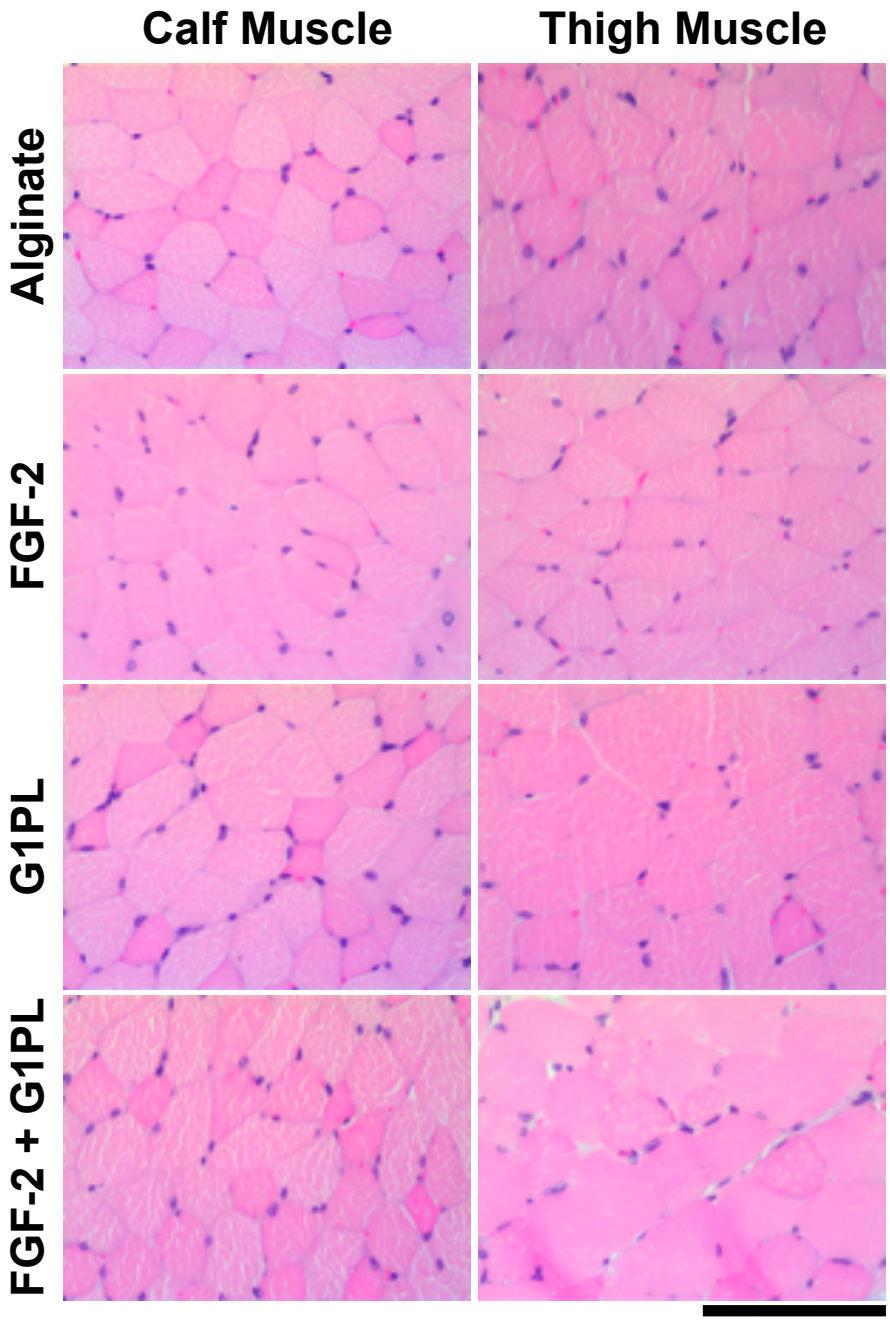

Figure 4.6 Representative H&E stained muscle fiber cross-sections of the calf and thigh of the ischemic limb.

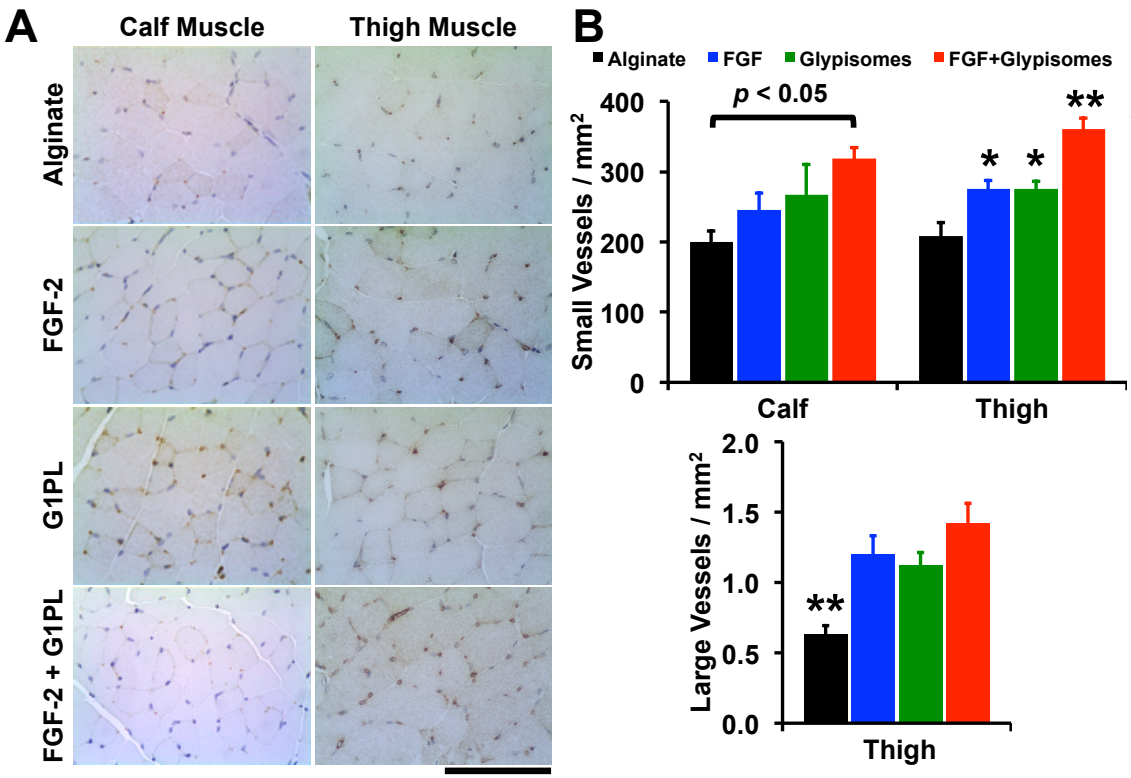

Figure 4.7 (A) Immunohistochemical staining for PECAM in calf and thigh muscle tissue of the ischemic limb. (B) Quantification of small vessel density in the calf and thigh muscle tissue, and large vessel density in the thigh muscle tissue. *Statistically significant difference from all other groups ($p < 0.05$). **Statistically significant difference from alginate group ($p < 0.05$). Bar = 100 μm.

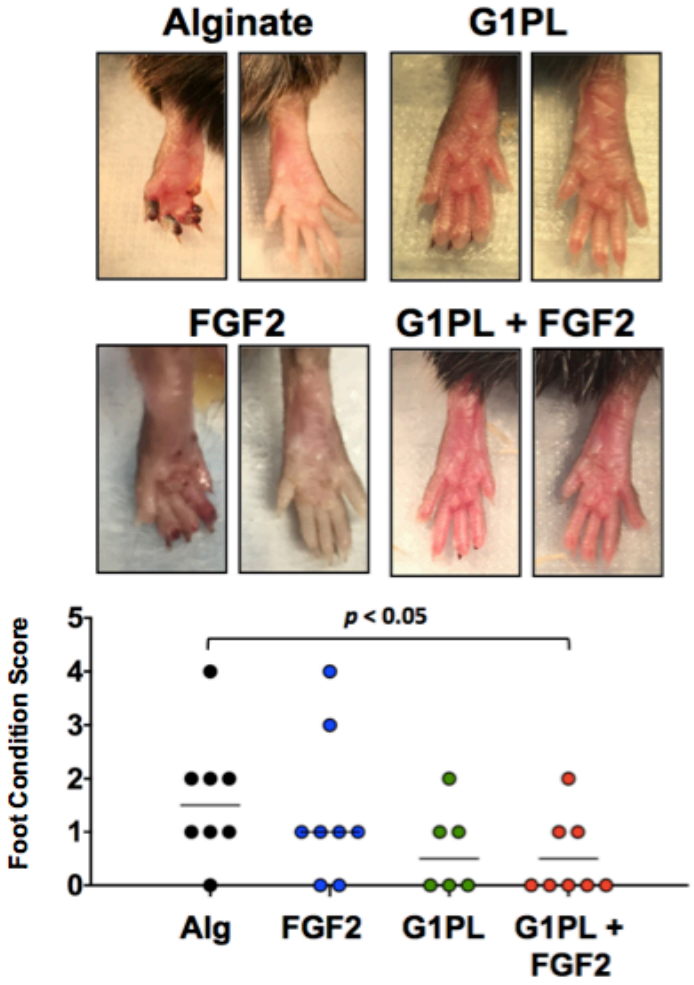

Figure 4.8 Glypisomes aid in preventing limb loss in an ob/ob hind limb ischemia model. Quantitative analysis of toe health at day 14 of the ob/ob hind limb ischemia study. Paws were scored from 0-4. A score of 0 indicates toes are normal, 1 indicates mouse had black toenails, 2 indicates a single black toe, 3 indicates multiple black toes, and 4 indicates that all toes are black. Bracket used to indicate significant difference ($p < 0.05$).

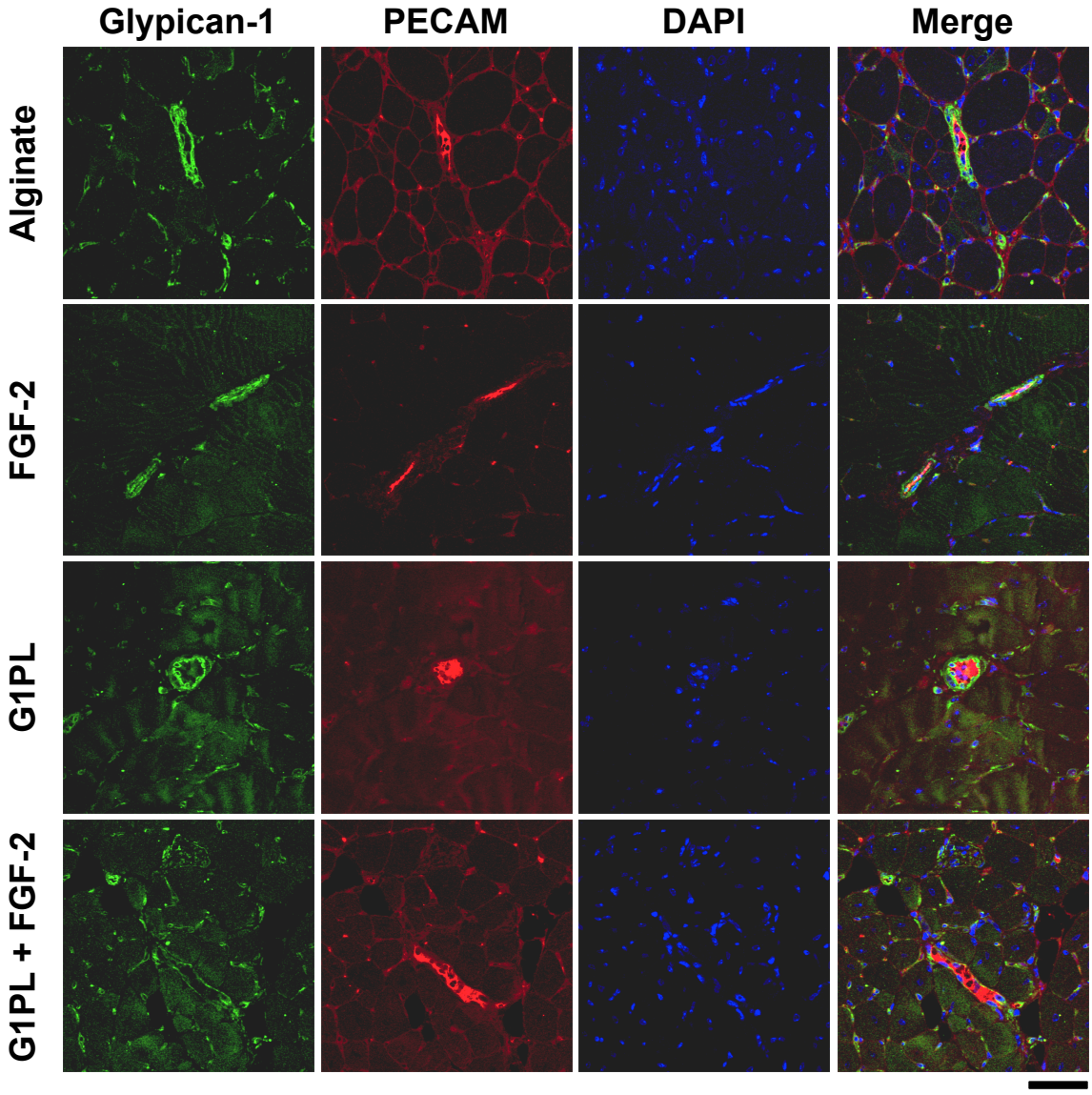

Figure 4.9 Delivery of glypisomes increases glypican-1 present in the muscle tissue of the ob/ob mice. Immunostaining for glypican-1 and PECAM in the calf muscles from the limbs of ob/ob mice that underwent femoral ligation. Bar = 50 μm.

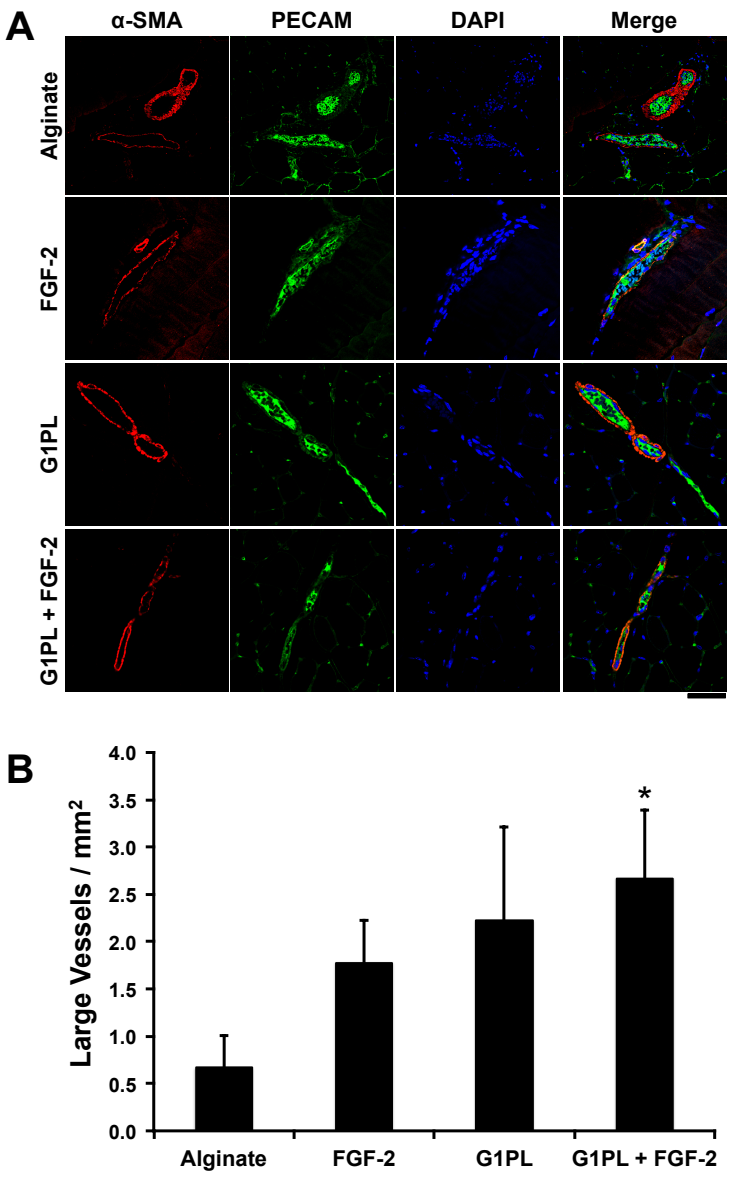

Figure 4.10 Glypisomes increase the number of α-SMA positive blood vessels following ischemic injury. (A) Immunostaining for α-SMA and PECAM in the thigh muscles from the limbs of ob/ob mice that underwent femoral ligation. (B) Quantification of a-SMA positive vessels in the thigh muscles from the ob/ob mice with femoral artery ligation. *$p < 0.05$ versus the alginate group. Bar = 50 μm.

4.4 DISCUSSION

We delivered the glypican-1 embedded in a liposome membrane to simulate the endogenous glypican-1 presentation and to facilitate uptake of the protein. The formulation is stable when encapsulated in alginate and can be delivered locally to ischemic areas of the body. We show here that this method is very effective in increasing FGF-2 activity when locally delivered to the ischemic limb in wild type or diabetic mice.

Our *in vivo* studies found that glypisomes enhanced growth factor activity in both wild type and ob/ob mouse models. In our studies, the perfusion of the hind limbs of the ob/ob mice recovered to a greater extent than wild type mice with treatment with FGF-2 and glypisomes. Depending on the disease state the diabetic mice may undergo ischemic conditioning (i.e. their disease induces ischemia which can cause the growth of collateral vessels). In this case, the high fat diet may serve to induce ischemic conditioning in the ob/ob mice, facilitating their recovery from femoral ligation.

In addition to its effects on angiogenesis, glypican-1 has been found to be important in the regulation of myogenic satellite cell proliferation and differentiation [40, 135, 136]. Critical limb ischemia is often found in severe cases of peripheral vascular disease and leads to muscle atrophy in addition to many other symptoms [137]. Our study found a marked decrease in ischemia-induced muscle fiber damage in glypisome treated mice. Thus, the strategy of exogenously delivering glypican-1 may act through both angiogenic and muscle preserving mechanisms to improve the response to FGF therapy in-vivo. Consequently, glypisomes may present a therapeutic for treating both the deleterious effects of peripheral ischemia and provide additional myoprotective benefits by providing increased levels of gypican-1 in the muscle.

4.5 CONCLUSIONS

In summary, our study supports that glypisomes can markedly improve the effectiveness of growth factor therapies in the context of ischemia in the diabetic disease state. This conceptual archetype of delivering lost co-receptors to replace molecular alterations due to disease may be relevant to many potential therapeutic applications in which an undesirable change in tissue biology makes current therapies untenable. From the poor or equivocal results of many clinical trials using growth factors it is clear that growth factor therapy alone is not sufficient to treat human disease in many cases. Therefore, therapeutic enhancers such as glypisomes are needed to truly address the complexities of growth factor responsiveness in the context of human disease.

Chapter 5: Glioblastoma Exosomes as a Therapy for Peripheral Limb Ischemia

5.1 Introduction

Exosomes are nanoparticles that are secreted by cells into their environment and range from approximately 30 to 100 nm in diameter [53]. These secreted extracellular vesicles are formed initially by formation of invaginations in the endosomal membrane to create a multivesicular body (MVB) [54]. This process is in contrast to that for microvesicles (200-1000 nm in diameter) that are formed by membrane shedding [55]. Exosomes are released through multivesicular endosomal fusion with the plasma membrane and are found in many biofluids. These secreted vesicles have now been recognized as mediators of cell-cell communication, capable of delivering molecules between distant cells[56]. Exosomes, in particular are known to have high levels of tetraspanins, trafficking/export-related molecules and heat shock proteins [57-60]. In addition, exosomes contain proteins, microRNA (miRNA) and, in some cases, double stranded DNA [61, 62]. In addition, exosomes carry bioactive lipids including sphingomyelin, eicosanoids, cholesterol and ganglioside GM3 [63]. Extracellular vesicles including exosomes are efficiently taken up by cells in culture where they modify the target cells transcriptional and protein expression profiles.

In particular, exosomes from many cell types have been known to mediate angiogenesis [64-69]. Exosomes derived from mesenchymal stem cells have also been shown to induce angiogenesis and modulate revascularization in ischemia and wound healing [68, 69]. Glioblastoma is one of the most angiogenic tumors, induce an intense growth of surrounding blood vessels [70]. The exosomes secreted by glioblastoma cells

prime endothelial cells to respond to hypoxic conditions with an angiogenic response [71]. Moreover, primary glioblastoma cells carry angiogenic miRNAs and proteins that facilitate angiogenic differentiation of endothelial cells [72]. Extracellular vesicles from glioblastoma cells have been shown to carry proteins that have both oncogenic and tumor suppressive activities including EGF receptors, PDGFR-A and PTEN [72-75]. Thus, while it is well supported that glioblastoma exosomes enhance angiogenesis it is less clear what role they play in tumor progression and metastasis. In addition, glioma-derived exosomes have immunomodulatory effects and drive macrophages towards the M2-phenotype [76]. Macrophages polarized towards the M2-phenotype secrete VEGF and promote angiogenesis [77, 78].

Exosomes are an emerging therapeutic strategy and have been explored as direct treatments for disease and drug carriers [79-82]. Exosomes from mesenchymal stem cells have generated significant interest and have been used to induce angiogenesis in wound healing and in ischemia [69, 83]. In addition, mesenchymal stem cell exosomes have improved the recovery following myocardial infarction and enhance cardiac regeneration [84, 85]. In this study, we aimed to examine using glioblastoma exosomes as therapeutics for enhancing revascularization in peripheral limb ischemia. Glioblastoma is one the most highly vascularized solid tumors and produces an intense angiogenic response. Moreover, the exosomes produced by these cells have been linked to growth promoting signals in endothelial cells [86]. Here, we explored whether this powerful aspect of tumor biology could be harnessed to enhance angiogenic therapies for patients with peripheral vascular disease.

5.2 MATERIALS AND METHODS

5.2.1 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Promocell, Inc. and glioblastoma cells (A-172) were purchased from ATCC. The HUVECs were cultured in MCDB-131 medium with 7.5% FBS, EGM-2 supplements (Lonza), L-glutamine and antibiotics. The glioblastoma cell line (A-172) was cultured in DMEM with 10% FBS, L-glutamine, and antibiotics. Both cell lines were grown at 37°C and in a 5% CO_2 atmosphere.

5.2.2 Purification of Exosomes

A glioblastoma cell line (A-172) was cultured to 60% confluence. Then cells were rinsed with PBS and the media was replaced with media containing exosome depleted FBS (20%) cultured for 48 hrs. The media was collected and exosomes were isolated from the media using the Exoquick-TC isolation kit (Systems Bioscience) and stored in PBS with magnesium in at -80°C. The number of exosomes was normalized to the exosome cell surface marker CD63 using a CD63 ELISA (Systems Bioscience). The number of exosomes was normalized to the exosome cell surface marker CD63 using a CD63 ELISA (Systems Bioscience).

5.2.3 Exosome Characterization

The size of the exosomes was measured using dynamic light scattering (Malvern Zetasizer Nano ZS). The instrument was calibrated using 54-nm diameter polystyrene particles and the exosomes were diluted in order to fit the detection region of the instrument. Final results were an average of 50 size measurements. For imaging the exosomes with cryo-electron microscopy, the samples were plunge-frozen in liquid ethane on carbon film grids as previously described (R2x2 Quantifoil; Micro Tools

GmbH, Jena, Germany).[138] The grids were then transferred to a cryo-specimen holder (Gatan 626) under liquid nitrogen and imaged using a transmission electron microscope (JEOL 2100 LaB6) operating at 200 keV. Grids were maintained at low temperatures during imaging session (-172°C to -180°C). The exosomes were imaged at 20,000x EM magnification with a 4000 x 4000 slow-scan CCD camera (UltraScan 895, GATAN, Inc.) using low-dose imaging procedure. ELISA's were used to quantify presence of glypican-1 (Ray Biotech) and syndecan-4 (Takara Clontech).

5.2.4 Proteomic Analysis with Mass Spectroscopy

Glioblastoma (A-172) cells were cultured to 70% confluence and then media was changed to exosome-depleted media. The media was collected 48 hours later and the exosomes were isolated using ExoQuick TC (Systems Bioscience). The exosomes were then processed with the XPEP Complete procedure. Nano LC/MS/MS was used to analyze the peptide libraries with a Waters NanoAcquity HPLC system and a ThermoFisher Q Exactive. The peptides were loaded on a trapping column and then eluted at 350nL/min using a 2 hr reverse phase gradient over a 75 μm analytical column. The columns were packed with Jupiter Proteo resin (Phenomenex). An injection volume of 30 μL was used. The mass spectrometer was used in data dependent mode, the orbitrap was set to 60,000 FWHM for MS and 17,500 FWHM for MS/MS.

5.2.5 Exosome RNA Sequencing

The glioblastoma cell line (A-172 cells) was grown to 70% confluence in 10 cm tissue culture plates. The media was changed to exosome-depleted media and cells were cultured for 48 hrs. The media was the collected and exosomes were isolated from the media using ExoQuick TC (Systems Bioscience). The total RNA was taken from the isolated exosomes using TRIzol. The quality of the isolated RNA was assessed using the

Agilent Bioanalyzer 2100 from the Functional Genomics Laboratory in the University of California in Berkeley. A RIN score of over 9 qualified the sample for cDNA production. To construct the library, one microgram of total RNA was used to isolate poly(A) purified mRNA. Average fragment sizes were 400 bp. Sequencing was done with an Ilumina HiSeq 2500 and each sample had 25-29 million 100-bp end reads. Read alignment was done by mapping to the mouse reference genome (UCSC/mm9) using Tophat [139], and HTSeq [140] was used to sum mapped reads for gene expression levels. DEseq [141] was used to normalize the read counts.

5.2.3 Proliferation Assay

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured 70% confluence and then media was changed to low serum media (2% FBS, no growth factors added) and incubated for 24 hrs. The cells were then passaged into a 96 well plate at 2500 cells/well and exosomes (4.4 or 30 * 10^8/ml) and/or FGF-2 (10 ng/ml) was added to the cells. BrdU was added to the cells 24 hrs post treatment. Then proliferation was assessed by BrdU incorporation at 12 hrs thereafter using a BrdU Assay (Cell Signaling).

5.2.3 In Vitro Angiogenesis Assay

The differentiation of endothelial cells was measured using an *in* vitro tube formation assay. Briefly, culture plates were coated with growth factor reduced matrigel at 37°C for 1 hour. In each well, 20,000 cells were seeded in the presence of different treatments with exosomes (4.4 or 30 * 10^8/ml) or FGF-2 (10 ng/ml). After 16 hours, the cells were imaged using phase contrast microscopy. Quantification of the number of branch points and tube length was performed using MetaMorph software (Molecular Devices).

5.2.5 Mouse Model of Hind Limb Ischemia

Wild type C57BL/6 mice were used in the hind limb ischemia studies. Wild type mice were maintained on a normal mouse chow diet. The mice were anesthetized with isofluorane gas and the femoral artery was exposed through an incision in the inguinal region. The artery was separated from the femoral nerve and vein, and then ligated in two locations using a 6-0 silk suture. Alginate beads containing the exosomes (15 billion exosomes/mouse) and FGF-2 (1.5 μg/mouse) were implanted in a total volume of 200 μl. The incision was then closed using degradable sutures. Relative blood flow between the ischemic and contralateral control limb was measured at days 1, 3, 5, 7, and 14 using laser speckle imaging as described previously.[132] Briefly, the hind paws of the mouse were illuminated by a 785 nm, 50 mW laser diode (Thor Labs) and imaged using a Zoom-7000 lnse (Navitar) and a Bassler CCD camera. Relative perfusion for the hind limb ischemia study was quantified and normalized to the contralateral limb as a control. At day 14, the mice were sacrificed and the tissues of the hind limb were harvested and frozen in liquid N_2 cooled isopentane. All animal procedures were approved by the Institutional Animal Care and Use Committee of UT Austin and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

5.2. Statistical Analysis

All results are shown as mean ± standard error of the mean. Comparisons between only two groups were performed using a 2-tailed Student's t-test. Differences were considered significant at $p < 0.05$. Multiple comparisons between groups were analyzed by 2-way ANOVA followed by a Tukey post-hoc test. A 2-tailed probability value $p < 0.05$ was considered statistically significant.

5.3 RESULTS

5.3.1 Characterization of Size and Morphology of Glioblastoma Exosomes

We isolated exosomes from the glioblastoma cell and analyzed them using cryo-electron microscopy. We found a heterogeneous mix of vesicles that were predominantly in the range of 30-100 nm but also included a significant portion of larger microvesicles and exosomes aggregates (Figure 5.1). A DLS analysis of the size distribution included two peaks with maximums of 28 and 164 nm (Figure 5.2). Our group has recently shown that delivery of syndecan-4 or glypican-1 containing vesicles enhances angiogenic growth factor therapy.[4, 127] We performed an ELISA for syndecan-4 and glypican-1 on the exosomes and found that there were high concentrations of both proteins in the isolated exosomes (Figure 5.3).

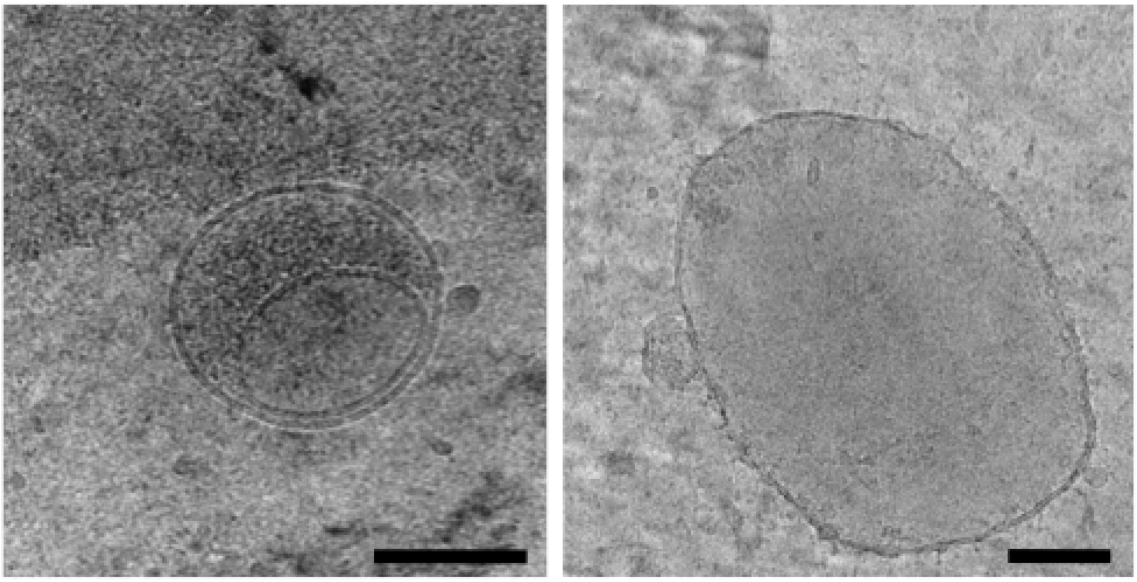

Figure 5.1 Characterization of exosomes isolated from glioblastoma cell line. Cryo-electron microscopy of exosomes. We observed heterogeneous mix of vesicles within the isolate, including exosomes and microvesicles. Bar = 100 nm.

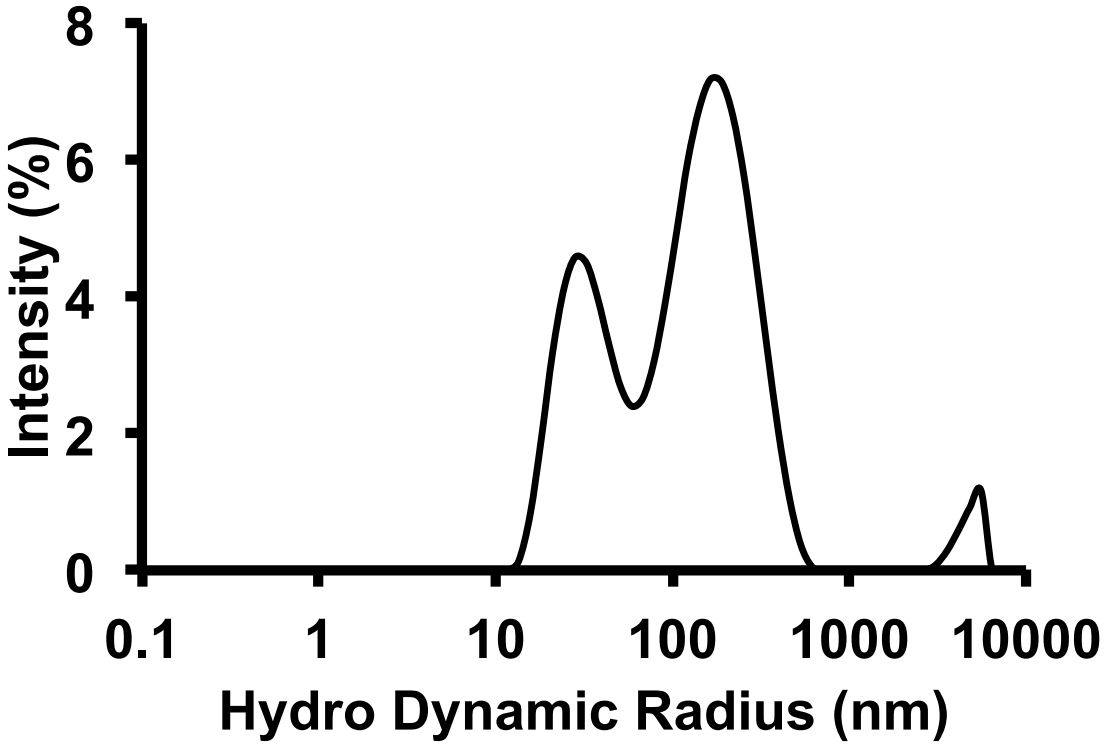

Figure 5.2 Dynamic light scattering analysis of isolated vesicles.

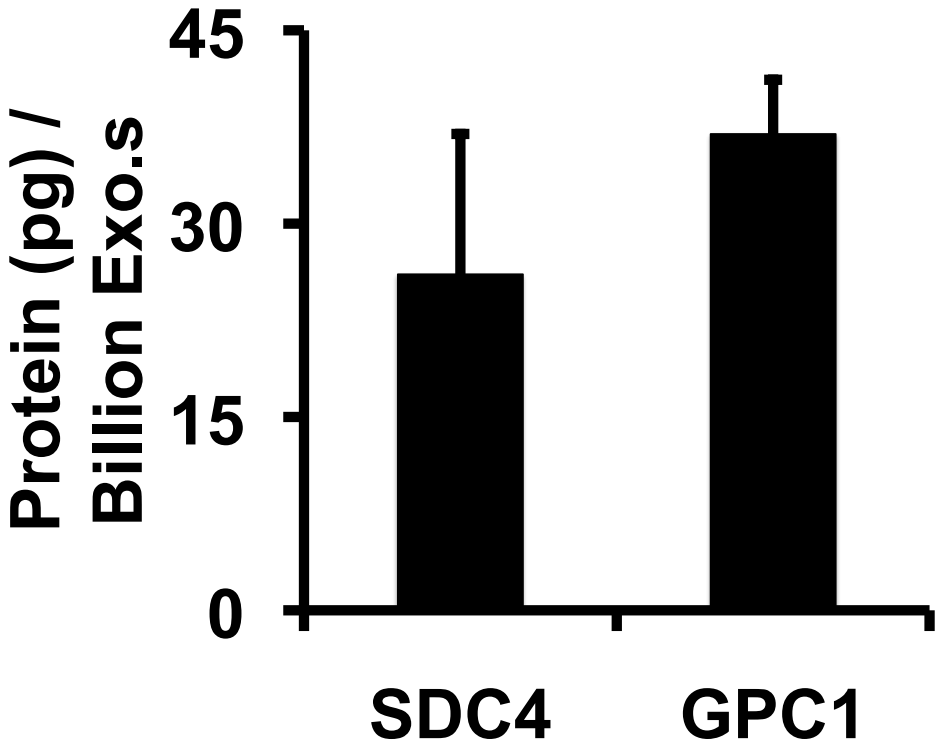

Figure 5.3 Concentration of syndecan-4 (SDC-4) and glypican-1 (GPC1) in exosomes isolated from glioblastoma cells quantified by ELISA and normalized to number of exosomes using CD63.

5.3.2 RNA expression and Proteomic Analysis of Exosomes

We ran next generation RNA sequencing on the exosome samples to look at the RNA content of the isolated exosomes and identified the top 25 most abundant miRNA (Figure 5.4A and Figure 5.4B). We then looked at the angiogenic potential of these miRNA and categorized their association with cancer proliferation and metastasis (Table 5.1). The most abundant was miR-221 which has been linked to new vascular formation[142], this particular miRNA was present at 3 fold higher levels than any other miRNA we detected (Figure 5.4B).

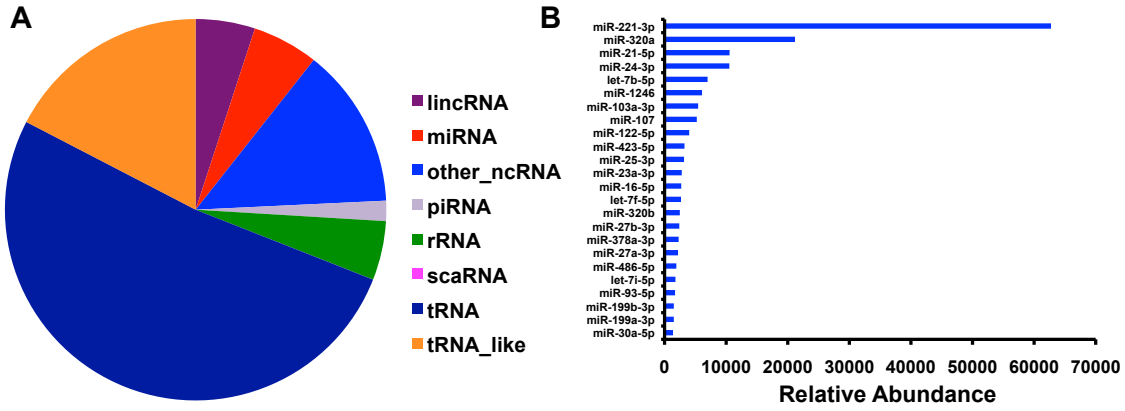

Figure 5.4 (A) Types of RNA present in the isolated exosome samples. (B) The top 25 most common miRNA detected in the isolated glioblastoma exosomes and their relative abundance.

Table 1, Most Abundant miRNA in Glioblastoma Exosomes

Abundance	miRNA	Function
62746.87	miR-221	Plays a role in new vessel formation[142, 143], upregulates proliferation[144] and migration[145]
21170.88	miR-320a	Inhibits cell proliferation and migration[146, 147] [147]
10553.53	miR-21	Encourages angiogenesis[148, 149] may be anti-oncogenic[150] promotes metastasis[151] and proliferation[152]
10512.92	miR-24	Downregulates cardiac tissue angiogenesis[153], upregulates tumor angiogenesis and apoptosis[154], upregulates proliferation/metastasis[155]
6984.64	Let-7b	Linked to reduced tumor angiogenesis[156], inhibits glioblastoma cell migration[157]
6080.81	miR-1246	Linked to increased angiogenesis[158] promotes growth and metastasis[159]
5493.73	Let-7a	Inhibits glioma malignancy[160]
5449.64	Mir-103	Upregulates VEGF, angiogenesis[161], metastasis[162] downregulates proliferation[163]
5231.52	miR-107	Upregulates angiogenesis post stroke[164], downregulates tumor angiogenesis[165]
4000.5	miR-122	Inhibits tumor proliferation[166] inhibits metastasis[167]
3233.59	mir-423	Upregulates proliferation[168]
3161.65	mir-25	Associated with glioma progression[169]
2786.89	mir-23a	Down regulates angiogenesis[170], increases glioma progression[171]
2703.36	mir-16	Suppresses angiogenesis[172], leads to apoptosis[173], inhibits metastasis[174]
5491.41	let-7f	Inhibits tumor proliferation[175], inhibits metastasis[176]
2477.11	mir-320b	Downregulates proliferation[177]
2391.25	mir-27b	Downregulates VEGF-C[178], upregulates tumor proliferation[179] and metastasis[180]
2284.51	miR-378a	Upregulates angiogenesis and tumor proliferation[181]
2178.93	miR-27a	Promotes proliferation and migration[182]
1909.75	mir-486	Promotes angiogenesis and proliferation[183]
1762.4	Let-7i	Leads to cell death[184]
1700.91	miR-93	Induces angiogenesis, proliferation, and migration[185]
1500.19	mir-199a	Downregulates angiogenesis[186] and proliferation[187]
1500.19	mir-199b	Promotes angiogenesis[188] promotes cell proliferation and migration[189]
1367.92	mir-30a	Promotes angiogenesis[190] and migration[191], can lead to apoptosis[192]

Table 5.1 Table of the 25 most abundant miRNA's in the isolated exosomes. Each miRNA's role in angiogenesis (if any), and their role in cancer proliferation or metastasis (if any) are also described.

5.3.3 Glioblastoma Exosomes Enhance Proliferation and Tube Formation in Endothelial Cells

We next examined whether exosomes could alter the behavior of endothelial cells in culture. We applied exosomes and exosomes with FGF-2 to cultured endothelial cells and measured their proliferation. We found that high levels of exosomes had synergistic activity with FGF-2 in increasing endothelial cell proliferation (Figure 5.5). We next tested the effect of exosomes in altering tube formation in endothelial cells. The exosomes increased the number of branch points and number of tubes formed at lower concentrations (Figure 5.6). In addition, the exosomes reduced the tube length of the network formed both with and without FGF-2 treatment.

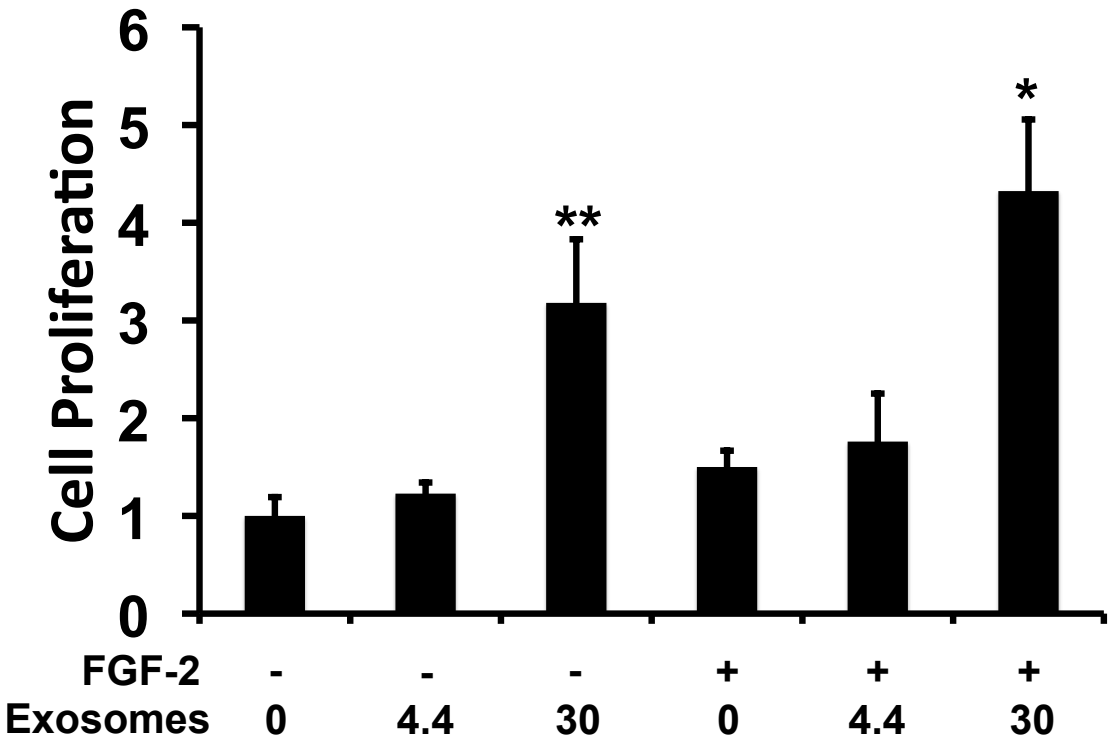

Figure 5.5 Glioblastoma exosomes increase endothelial cell proliferation. Proliferation of endothelial cells measured by BrdU incorporation after 24 hours of treatment with 10 ng/ml FGF-2 and/or exosomes (10^8/ml). *Statistically significant different from groups treated with low concentration of exosomes with and without FGF-2 ($p < 0.05$). **Statistically significant difference from the control ($p < 0.05$).

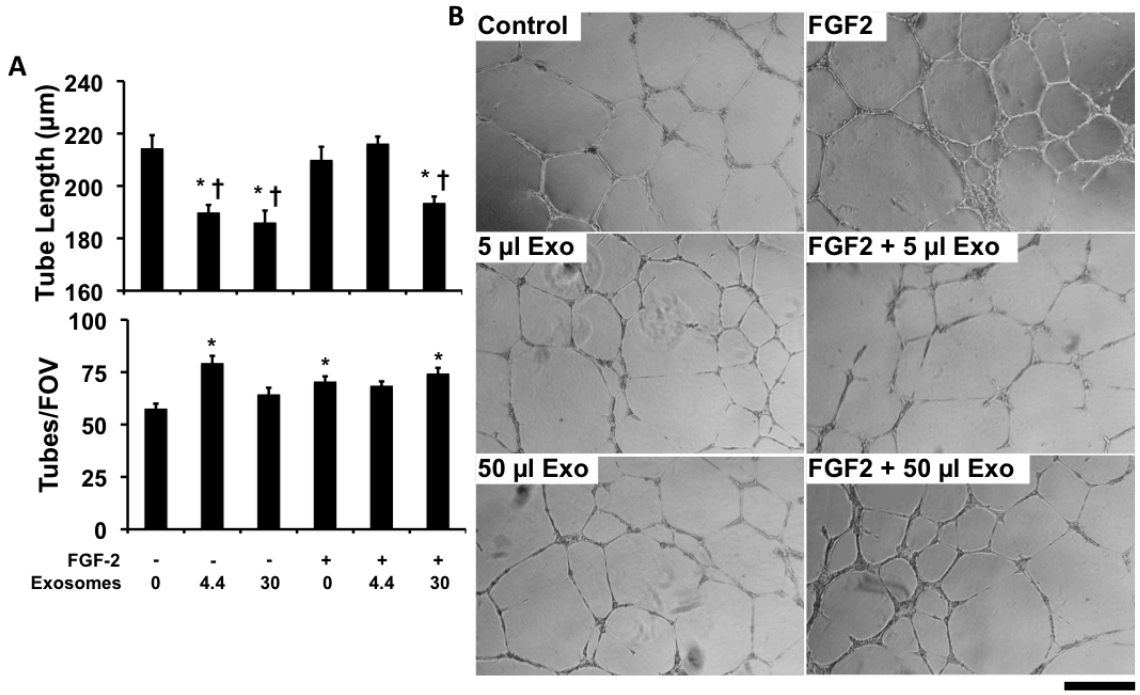

Figure 5.6 Glioblastoma exosomes reduce tube length in tube formation assay (A) Quantification of tube length and tubes per field of view (B) Endothelial cells were grown on matrigel and treated with exosomes (10^8/ml) and/or 10 ng/ml FGF-2. After 16 hours the formation of tubes was assessed by phase contrast microscopy. *Statistically significant difference from control group ($p < 0.05$). †Statistically significant difference from FGF-2 and FGF-2 with low exosome dose groups ($p < 0.05$). Bar = 200 μm.

5.3.4 Local Delivery of Glioblastoma Exosomes Enhances Therapeutic Angiogenesis in the Ischemic Hind Limb of Mice

The delivery of FGF-2 enhances revascularization in animal models if ischemia.[4, 127] Our group has recently shown that delivering proteoliposomes that contain syndecan-4 or glypican-1 in combination with growth factors markedly improves revascularization in health and diabetic animals.[4, 127] We created ischemia in the hind limb of mice by ligating the femoral artery and implanted alginate beads with FGF-2 or FGF-2 and exosomes (Figure 5.7A and Figure 5.7B). We monitored perfusion in hind limbs for 14 days. Exosomes enhanced the recovery of perfusion in the hind limbs, with the exosomes and FGF-2 restoring approximately 75% of the perfusion relative to the control limb after 14 days (Figure 5.8A and Figure 5.8D). We found that there was a reduction in the ischemic muscle fiber changes (Figure 5.9). In addition, we immunostained for the vessels within the muscles and found increased capillary density in the thigh and calf muscles, consistent with the increased perfusion observed by laser speckle imaging (Figure 5.10A and Figure 5.10B). An analysis of larger vessels revealed increased arteriogenesis in the thigh muscle of exosomes-treated mice (Figure 5.10C).

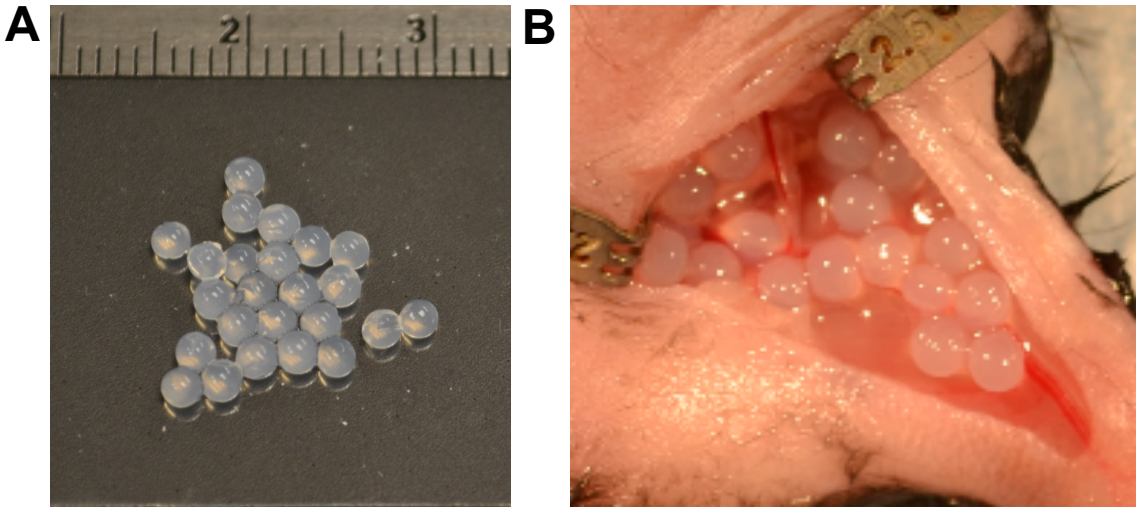

Figure 5.7 Glioblastoma exosomes enhance therapeutic angiogenesis with FGF-2 in hind limb ischemia. (A) Glioblastoma exosomes were encapsulated in alginate beads. (B) Ischemia was induced in mice through femoral artery ligation and the alginate beads were implanted at the time of surgery.

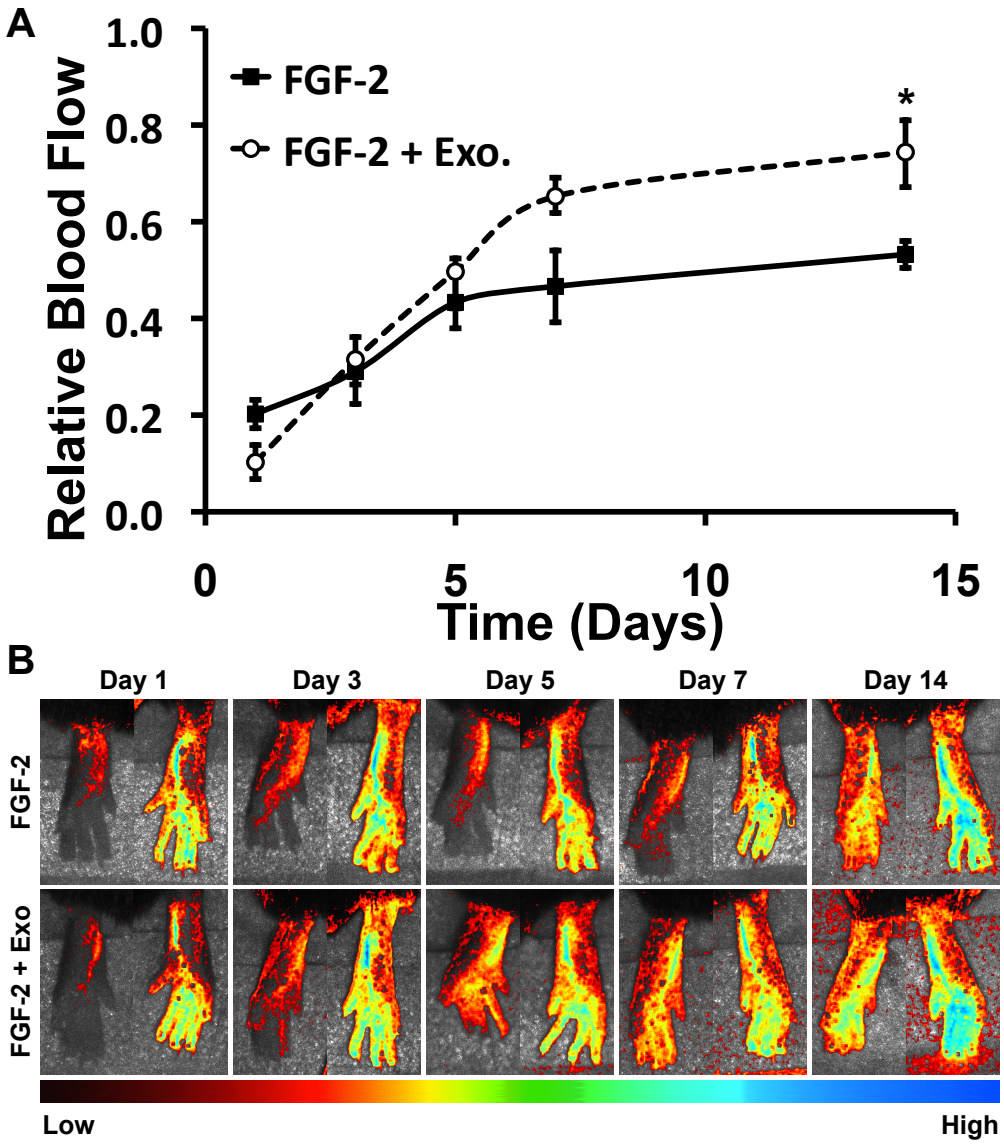

Figure 5.8 Glioblastoma exosomes lead to increased recovered relative perfusion in a mouse hind limb ischemia model. (A) Perfusion of the feet after induction of hind limb ischemia. Relative blood flow was defined as the ratio between the ischemic limb and the control limb. *Statistically significant difference with the FGF-2 alone group at the same time point ($p < 0.05$). (B) Laser speckle contrast imaging analysis of blood perfusion in the feet of the mice over time. Mice were given either alginate beads with FGF-2 or FGF-2 with glioblastoma exosomes.

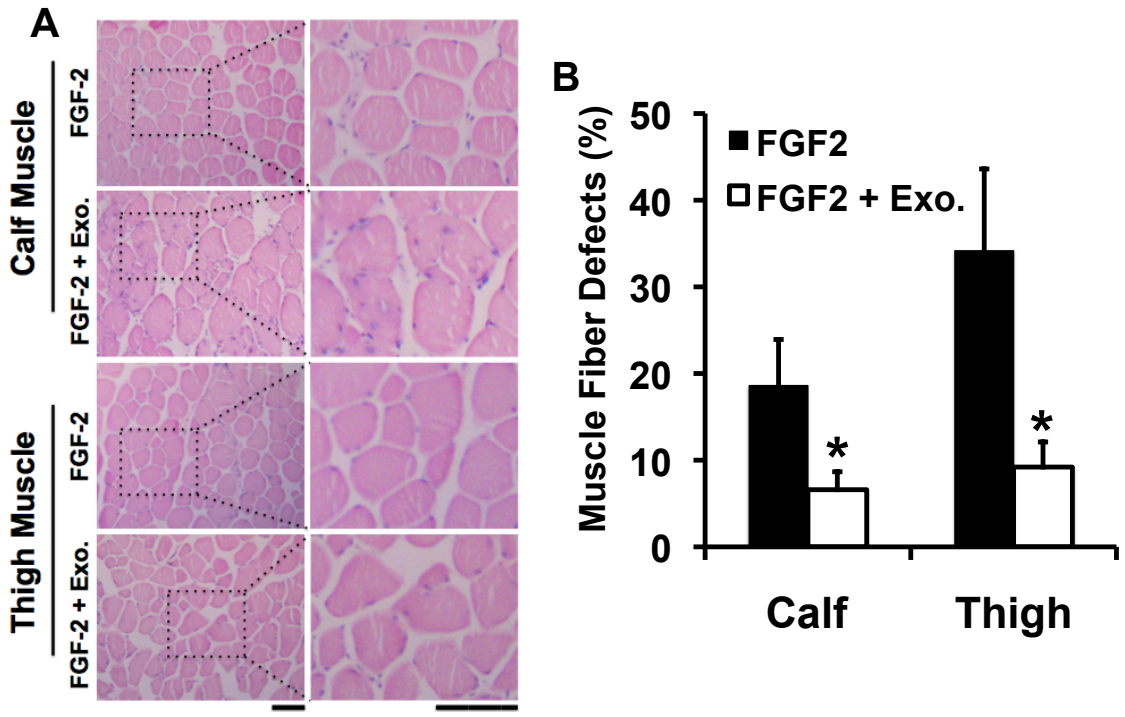

Figure 5.9 Exosomes reduce muscle damage and enhance vascularity of the ischemic hind limb in mice. (A) Histological sections from the hind limb of mice with femoral artery ligation. (B) Ischemic changes in histology were reduced in the calf muscle with FGF-2 and exosomes treatment in comparison to FGF-2 alone. *Statistically significant difference with FGF-2 alone group ($p < 0.05$). Bar = 100 μm.

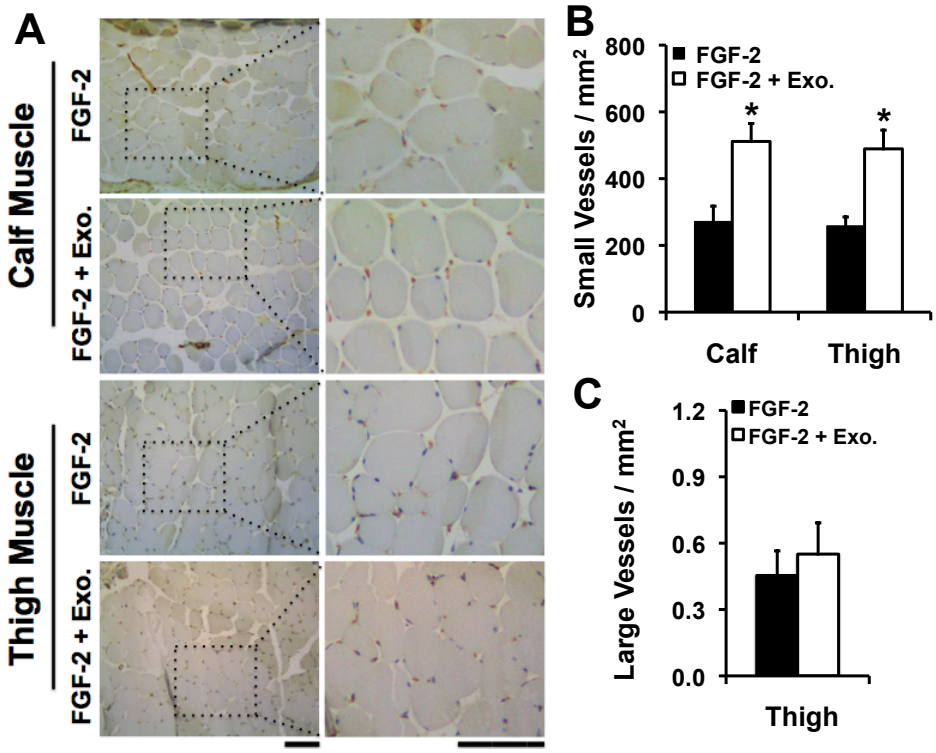

Figure 5.10 Exosomes lead to an increased (A) Immunohistochemical staining for PECAM-1 (blood vessels) in the muscles of the limb. (B) Quantification of small vessel density within the muscles. (C) Quantification of large vessel density (diameter > 25 μm) within the muscles. *Statistically significant difference with FGF-2 alone group ($p < 0.05$). Bar = 100 μm.

5.4 DISCUSSION

Exosomes are emerging therapeutics with applications ranging from cancer to cardiovascular disease. Several groups have shown that are benefits from exosomes from mesenchymal stem cells and, indeed, these effects may underlie the benefits of cell therapy in clinical trials. Tumors require an ever-increasing vascular supply to maintain their rapid growth within the native tissue. In particular, glioblastomas show intense angiogenesis that maintains their growth within the brain. Here, we aimed to test if the strong pro-angiogenic properties of tumor exosomes could be harnessed to treat ischemia in peripheral vascular disease. In the past, angiogenic therapies have not fared well in clinical trials. Thus, a robust angiogenic stimulus from multiple sources within tumor exosomes may provide benefit where a single growth factor or gene would not. Here, we show that exosomes from glioblastoma can effectively induce angiogenesis in the context of ischemia. In particular, exosomes provided an angiogenic signal that potentiated the therapeutic potential of FGF-2 both in *in vitro* and *in vivo* assays of angiogenesis.

Our analysis of the content of glioblastoma revealed that the most abundant miRNA found in our isolated glioblastoma exosomes was miR-221, which has been shown to play an important role in the formation of new blood vessels [142]. This miRNA has been linked specifically to angiogenesis in glioblastoma [193] and increased glioblastoma cell proliferation [144]. Of the 25 most common miRNAs present in the isolated exosomes, nine have been shown to promote angiogenesis while only four have been shown to inhibit angiogenic pathways. Interestingly many of the miRNA also have been shown to promote cell proliferation and metastasis.

Together these signals constitute a robust and multi-pathway angiogenic stimulus. In addition, our group has recently shown that proteoliposomes carrying proteoglycans, including both syndecan-4 and glypican-1, are highly effective in enhancing growth factor therapies [4, 127]. This is particularly important for inducing therapeutic angiogenesis in disease states such as diabetes, where there is a loss in these critical growth factor receptors [4]. Our results suggest that exosomes act in mechanisms in addition to direct stimulation of angiogenesis in endothelial cells. Our results from the

tube formation assay show an increase in branch points with exosomes treatment but a reduction in tube length. This may suggest that exosomes induce very dense vascular network formation. In our study, the exosomes provided intense growth signals to endothelial cells. *In vivo*, the exosomes enhanced FGF-2 induced revascularization. We found, however, that this result was primarily from capillary growth in calf muscles rather than arteriogenesis. This is consistent with our findings in the tube formation assay that showed small capillary-like branching.

5.5 CONCLUSIONS

We have shown that glioblastoma exosomes have potential as therapeutics for ischemia. Our studies support that the exosomes strongly induce angiogenesis *in vivo* and contain a host of pro-angiogenic signals that act through multiple mechanisms. This would likely be highly beneficial as treatments for severe ischemia given the presence therapeutic resistance in long-term disease that may prevent efficacy of single growth factors or genes. Thus, cancer derived exosomes as therapeutics may be applicable to many disease states and conditions that would benefit form enhanced capillary formation.

Chapter 6: Conclusions and Future Work

6.1 Conclusions

We have shown that glypican-1 is down regulated in the long-term disease state associated with peripheral limb ischemia. We have demonstrated that co-delivering growth factors with glypisomes also leads to greater angiogenic response *in vitro* by increasing endothelial proliferation, migration and tube formation. We have found evidence that this response may be due to increased uptake and increased AKT signaling.

We then demonstrated the effectiveness of glypisomes co-delivered with FGF-2 to increase the recovered relative perfusion, using a hind limb ischemia model, in both wild type and disease model mice. We also saw an increase of large vessel and small vessel density both the calf and thigh muscle tissue in these mice. In the disease model we saw a significant decrease in toe damage following the induced ischemia as a result of co-delivering FGF-2 and the glypisomes, suggesting that they are an effective therapy for peripheral limb ischemia.

Finally we isolated exosomes from glioblastoma, and found that they contained glypican-1 and syndecan-4, which have been shown to enhance growth factor activity [4, 194]. We also found that they contain many proangiogenic miRNA. When co-delivering these exosomes with FGF-2 we see a significant increase in endothelial proliferation. We saw that in a wild type hind limb ischemia model, co-delivering exosomes and FGF-2 leads to an increase in recovered relative perfusion following the femoral ligation injury. This treatment also lead to an increase in vascular density and a reduction of muscle fiber damages. This suggests that glioblastoma exosomes may be an appealing treatment for therapeutic angiogenesis.

6.2 FUTURE WORK

6.1 Intracellular Trafficking of Glypican-1

To further understand why we see a greater angiogenic response to the glypisomes we will look at endosomal trafficking of the delivered glypican-1. To do this we will use the constructs for expressing GFP-Rab5, GFP-Rab7 and GFP-Rab11 (early, mid and late endosomal markers) described previously [195]. To study the effect of glypisomes on endosomal trafficking, HEK 293 cells will be transfected with the constructs using lipofectamine 2000 (Life Technologies, inc.) to express each of these GFP labeled Rab proteins. We will conjugate Alexa Fluor 594 to glypican-1 using a kit (ThermoFischer) to label these proteins in solution and then re-isolate them using the his-tag on the labeled glypican-1. Then, these Alexa Fluor 594 labeled glypican-1 proteins will be incorporated into our glypisomes. Twenty-four hours after transfection, we will add the glypisomes and/or FGF-2 to the cells and then fix them at various time points. Using confocal microscopy we will image the cells and the percentage Rab-positive endosomes that are also glypican-1 positive will be quantified using 10 cells at each time. We also plan to repeat these experiments but rather than labeling FGF-2 with Alexa Fluor 594 we plan to label the glypican-1 used for the glypisomes. .

6.2 Wound Healing

Determine therapeutic potential for wound healing in a disease model mouse excisional wound model. To assess glypisomes as a potential therapeutic for non-healing diabetic foot ulcers we should use an excisional wound model with ob/ob mice as the disease model. A To prevent wound healing contraction I propose the use of the silicon splint wound healing model. Here a wound is created in the back of the mouse using a

biopsy punch and then a silicon splint of the same size is adhered to the surrounding skin and sutured into place. Alginate disks, containing either glypisomes and/or growth factors, would then be inserted in the wound bed. Over the course of two weeks we would monitor the wound healing process. On day 14 the mouse will be sacrificed and the wound will be biopsied and cut in half. One half will be fixed in 10% formalin for paraffin-embedded sectioning and the other half will be either snap frozen for later processing. Sections will be stained for M1 and M2 Macrophages (CD86 and CD163 respectively), vascularity (PECAM-1), and cytokeritin to assess wound healing. If we see a favorable response we will repeat this experiment, however wounds will be excised at days 2 and 6 to allow us to characterize the temporal behavior of the immune response. Half of the wound will be digested for FACS analysis of M1 and M2 macrophages at each time point.

My hypothesis is that co-delivery of glypisomes and growth factors will significantly increase wound-healing time. I expect that these wounds will close faster and show greater vascularization and re-epithelialization on day 14. I expect the wounds treated with both glypisomes and growth factors to also have a higher ratio of M2/M1 macrophages than wounds treated with growth factors alone and to transition from M1 to M2 macrophages at a faster rate. This shows a healthy, faster wound healing process.

References

[1] M. E. O'Donnell, J. A. Reid, L. L. Lau, R. J. Hannon, and B. Lee, "Optimal management of peripheral arterial disease for the non-specialist," *The Ulster medical journal,* vol. 80, pp. 33-41, 2011-Jan 2011.

[2] A. Grochot-Przeczek, J. Dulak, and A. Jozkowicz, "Therapeutic angiogenesis for revascularization in peripheral artery disease," *Gene,* vol. 525, pp. 220-228, Aug 10 2013.

[3] B. H. Annex, "Therapeutic angiogenesis for critical limb ischaemia," *Nature Reviews Cardiology,* vol. 10, pp. 387-396, Jul 2013.

[4] S. Das, G. Singh, and A. B. Baker, "Overcoming disease-induced growth factor resistance in therapeutic angiogenesis using recombinant co-receptors delivered by a liposomal system," *Biomaterials,* vol. 35, pp. 196-205, Jan 2014.

[5] L. Norgren, W. R. Hiatt, J. A. Dormandy, M. R. Nehler, K. A. Harris, F. G. Fowkes, *et al*., "Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II)," *J Vasc Surg,* vol. 45 Suppl S, pp. S5-67, Jan 2007.

[6] J. E. Udelson, V. Dilsizian, R. J. Laham, N. Chronos, J. Vansant, M. Blais, *et al*., "Therapeutic angiogenesis with recombinant fibroblast growth factor-2 improves stress and rest myocardial perfusion abnormalities in patients with severe symptomatic chronic coronary artery disease," *Circulation,* vol. 102, pp. 1605-10, Oct 3 2000.

[7] R. J. Lederman, F. O. Mendelsohn, R. D. Anderson, J. F. Saucedo, A. N. Tenaglia, J. B. Hermiller, *et al*., "Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial," *Lancet,* vol. 359, pp. 2053-8, Jun 15 2002.

[8] R. J. Lederman, A. N. Tenaglia, R. D. Anderson, J. B. Hermiller, K. Rocha-Singh, F. O. Mendelsohn, *et al*., "Design of the therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (TRAFFIC) trial," *Am J Cardiol,* vol. 88, pp. 192-5, A6-7, Jul 15 2001.

[9] S. Rajagopalan, E. R. Mohler, 3rd, R. J. Lederman, F. O. Mendelsohn, J. F. Saucedo, C. K. Goldman, *et al*., "Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication," *Circulation,* vol. 108, pp. 1933-8, Oct 21 2003.

[10] C. L. Grines, M. W. Watkins, G. Helmer, W. Penny, J. Brinker, J. D. Marmur, *et al*., "Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris," *Circulation,* vol. 105, pp. 1291-7, Mar 19 2002.

[11] Y. H. Kusumanto, V. van Weel, N. H. Mulder, A. J. Smit, J. J. van den Dungen, J. M. Hooymans, *et al.*, "Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial," *Hum Gene Ther,* vol. 17, pp. 683-91, Jun 2006.

[12] A. J. Comerota, R. C. Throm, K. A. Miller, T. Henry, N. Chronos, J. Laird, *et al.*, "Naked plasmid DNA encoding fibroblast growth factor type 1 for the treatment of end-stage unreconstructible lower extremity ischemia: preliminary results of a phase I trial," *J Vasc Surg,* vol. 35, pp. 930-6, May 2002.

[13] J. Belch, W. R. Hiatt, I. Baumgartner, I. V. Driver, S. Nikol, L. Norgren, *et al.*, "Effect of fibroblast growth factor NV1FGF on amputation and death: a randomised placebo-controlled trial of gene therapy in critical limb ischaemia," *Lancet,* vol. 377, pp. 1929-37, Jun 4 2011.

[14] H. Shigematsu, K. Yasuda, T. Iwai, T. Sasajima, S. Ishimaru, Y. Ohashi, *et al.*, "Randomized, double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical limb ischemia," *Gene Ther,* vol. 17, pp. 1152-61, Sep 2010.

[15] M. A. Creager, J. W. Olin, J. J. Belch, G. L. Moneta, T. D. Henry, S. Rajagopalan, *et al.*, "Effect of hypoxia-inducible factor-1alpha gene therapy on walking performance in patients with intermittent claudication," *Circulation,* vol. 124, pp. 1765-73, Oct 18 2011.

[16] M. Simons, B. H. Annex, R. J. Laham, N. Kleiman, T. Henry, H. Dauerman, *et al.*, "Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial," *Circulation,* vol. 105, pp. 788-93, Feb 19 2002.

[17] T. D. Henry, B. H. Annex, G. R. McKendall, M. A. Azrin, J. J. Lopez, F. J. Giordano, *et al.*, "The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis," *Circulation,* vol. 107, pp. 1359-65, Mar 18 2003.

[18] B. H. Annex, "Therapeutic angiogenesis for critical limb ischaemia," *Nat Rev Cardiol,* vol. 10, pp. 387-96, May 14 2013.

[19] "Effect of intensive diabetes management on macrovascular events and risk factors in the Diabetes Control and Complications Trial," *Am J Cardiol,* vol. 75, pp. 894-903, May 1 1995.

[20] G. Chen, D. Wang, R. Vikramadithyan, H. Yagyu, U. Saxena, S. Pillarisetti, *et al.*, "Inflammatory cytokines and fatty acids regulate endothelial cell heparanase expression," *Biochemistry,* vol. 43, pp. 4971-7, May 4 2004.

[21] F. Wang, M. S. Kim, P. Puthanveetil, G. Kewalramani, S. Deppe, S. Ghosh, *et al.*, "Endothelial heparanase secretion after acute hypoinsulinemia is regulated by glucose and fatty acid," *Am J Physiol Heart Circ Physiol,* vol. 296, pp. H1108-16, Apr 2009.

[22] D. Zhang, A. Wan, A. P. Chiu, Y. Wang, F. Wang, K. Neumaier, *et al.*, "Hyperglycemia-Induced Secretion of Endothelial Heparanase Stimulates a Vascular Endothelial Growth Factor Autocrine Network in Cardiomyocytes That

Promotes Recruitment of Lipoprotein Lipase," *Arterioscler Thromb Vasc Biol,* Oct 10 2013.

[23] A. B. Baker, Y. S. Chatzizisis, R. Beigel, M. Jonas, B. V. Stone, A. U. Coskun, *et al.*, "Regulation of heparanase expression in coronary artery disease in diabetic, hyperlipidemic swine," *Atherosclerosis,* vol. 213, pp. 436-42, Dec 2010.

[24] C. Osterholm, L. Folkersen, M. Lengquist, F. Ponten, T. Renne, J. Li, *et al.*, "Increased expression of heparanase in symptomatic carotid atherosclerosis," *Atherosclerosis,* vol. 226, pp. 67-73, Jan 2013.

[25] M. Blich, A. Golan, G. Arvatz, A. Sebbag, I. Shafat, E. Sabo, *et al.*, "Macrophage activation by heparanase is mediated by TLR-2 and TLR-4 and associates with plaque progression," *Arterioscler Thromb Vasc Biol,* vol. 33, pp. e56-65, Feb 2013.

[26] A. B. Baker, A. Groothuis, M. Jonas, D. S. Ettenson, T. Shazly, E. Zcharia, *et al.*, "Heparanase alters arterial structure, mechanics, and repair following endovascular stenting in mice," *Circ Res,* vol. 104, pp. 380-7, Feb 13 2009.

[27] A. B. Baker, W. J. Gibson, V. B. Kolachalama, M. Golomb, L. Indolfi, C. Spruell, *et al.*, "Heparanase regulates thrombosis in vascular injury and stent-induced flow disturbance," *J Am Coll Cardiol,* vol. 59, pp. 1551-60, Apr 24 2012.

[28] I. Vlodavsky, Y. Friedmann, M. Elkin, H. Aingorn, R. Atzmon, R. Ishai-Michaeli, *et al.*, "Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis," *Nat Med,* vol. 5, pp. 793-802, Jul 1999.

[29] M. D. Hulett, C. Freeman, B. J. Hamdorf, R. T. Baker, M. J. Harris, and C. R. Parish, "Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis," *Nat Med,* vol. 5, pp. 803-9, Jul 1999.

[30] A. Purushothaman, T. Uyama, F. Kobayashi, S. Yamada, K. Sugahara, A. C. Rapraeger, *et al.*, "Heparanase-enhanced shedding of syndecan-1 by myeloma cells promotes endothelial invasion and angiogenesis," *Blood,* vol. 115, pp. 2449-57, Mar 25 2010.

[31] Y. Yang, V. Macleod, H. Q. Miao, A. Theus, F. Zhan, J. D. Shaughnessy, Jr., *et al.*, "Heparanase enhances syndecan-1 shedding: a novel mechanism for stimulation of tumor growth and metastasis," *J Biol Chem,* vol. 282, pp. 13326-33, May 4 2007.

[32] M. G. Paulick and C. R. Bertozzi, "The glycosylphosphatidylinositol anchor: A complex membrane-anchoring structure for proteins," *Biochemistry,* vol. 47, pp. 6991-7000, Jul 2008.

[33] M. Murakami, T. Kambe, S. Shimbara, S. Yamamoto, H. Kuwata, and I. Kudo, "Functional association of type IIA secretory phospholipase A(2) with the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan in the cyclooxygenase-2-mediated delayed prostanoid-biosynthetic pathway," *J Biol Chem,* vol. 274, pp. 29927-36, Oct 15 1999.

[34] E. Marois, A. Mahmoud, and S. Eaton, "The endocytic pathway and formation of the Wingless morphogen gradient," *Development,* vol. 133, pp. 307-17, Jan 2006.

[35] A. Gallet, L. Staccini-Lavenant, and P. P. Therond, "Cellular trafficking of the glypican Dally-like is required for full-strength Hedgehog signaling and wingless transcytosis," *Dev Cell,* vol. 14, pp. 712-25, May 2008.

[36] K. Beckett, X. Franch-Marro, and J. P. Vincent, "Glypican-mediated endocytosis of Hedgehog has opposite effects in flies and mice," *Trends Cell Biol,* vol. 18, pp. 360-3, Aug 2008.

[37] H. C. Christianson, K. J. Svensson, T. H. van Kuppevelt, J. P. Li, and M. Belting, "Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity," *Proc Natl Acad Sci U S A,* vol. 110, pp. 17380-5, Oct 22 2013.

[38] J. Ledin, W. Staatz, J. P. Li, M. Gotte, S. Selleck, L. Kjellen*, et al*., "Heparan sulfate structure in mice with genetically modified heparan sulfate production," *J Biol Chem,* vol. 279, pp. 42732-41, Oct 8 2004.

[39] J. Kreuger, L. Perez, A. J. Giraldez, and S. M. Cohen, "Opposing activities of Dally-like glypican at high and low levels of Wingless morphogen activity," *Dev Cell,* vol. 7, pp. 503-12, Oct 2004.

[40] J. Gutierrez, D. Cabrera, and E. Brandan, "Glypican-1 regulates myoblast response to HGF via Met in a lipid raft-dependent mechanism: effect on migration of skeletal muscle precursor cells," *Skelet Muscle,* vol. 4, p. 5, 2014.

[41] A. Chamorro-Jorganes, E. Araldi, N. Rotllan, D. Cirera-Salinas, and Y. Suarez, "Autoregulation of glypican-1 by intronic microRNA-149 fine tunes the angiogenic response to FGF2 in human endothelial cells," *J Cell Sci,* vol. 127, pp. 1169-78, Mar 15 2014.

[42] P. P. Dwivedi, R. H. Grose, J. Filmus, C. S. Hii, C. J. Xian, P. J. Anderson*, et al*., "Regulation of bone morphogenetic protein signalling and cranial osteogenesis by Gpc1 and Gpc3," *Bone,* vol. 55, pp. 367-76, Aug 2013.

[43] J. Kleeff, T. Ishiwata, A. Kumbasar, H. Friess, M. W. Buchler, A. D. Lander*, et al*., "The cell-surface heparan sulfate proteoglycan glypican-1 regulates growth factor action in pancreatic carcinoma cells and is overexpressed in human pancreatic cancer," *J Clin Invest,* vol. 102, pp. 1662-73, Nov 1 1998.

[44] G. Su, K. Meyer, C. D. Nandini, D. Qiao, S. Salamat, and A. Friedl, "Glypican-1 is frequently overexpressed in human gliomas and enhances FGF-2 signaling in glioma cells," *Am J Pathol,* vol. 168, pp. 2014-26, Jun 2006.

[45] D. Qiao, K. Meyer, C. Mundhenke, S. A. Drew, and A. Friedl, "Heparan sulfate proteoglycans as regulators of fibroblast growth factor-2 signaling in brain endothelial cells. Specific role for glypican-1 in glioma angiogenesis," *J Biol Chem,* vol. 278, pp. 16045-53, May 2 2003.

[46] A. D. Theocharis, S. S. Skandalis, G. N. Tzanakakis, and N. K. Karamanos, "Proteoglycans in health and disease: novel roles for proteoglycans in malignancy and their pharmacological targeting," *FEBS J,* vol. 277, pp. 3904-23, Oct 2010.

[47] D. Qiao, K. Meyer, and A. Friedl, "Glypican 1 stimulates s phase entry and DNA replication in human glioma cells and normal astrocytes," *Mol Cell Biol,* vol. 33, pp. 4408-21, Nov 2013.

[48] S. Gengrinovitch, B. Berman, G. David, L. Witte, G. Neufeld, and D. Ron, "Glypican-1 is a VEGF165 binding proteoglycan that acts as an extracellular chaperone for VEGF165," *J Biol Chem,* vol. 274, pp. 10816-22, Apr 16 1999.

[49] Y. H. Jen, M. Musacchio, and A. D. Lander, "Glypican-1 controls brain size through regulation of fibroblast growth factor signaling in early neurogenesis," *Neural Dev,* vol. 4, p. 33, 2009.

[50] A. Fico, F. Maina, and R. Dono, "Fine-tuning of cell signaling by glypicans," *Cell Mol Life Sci,* vol. 68, pp. 923-9, Mar 2011.

[51] T. Aikawa, C. A. Whipple, M. E. Lopez, J. Gunn, A. Young, A. D. Lander, *et al.*, "Glypican-1 modulates the angiogenic and metastatic potential of human and mouse cancer cells," *J Clin Invest,* vol. 118, pp. 89-99, Jan 2008.

[52] D. Qiao, K. Meyer, and A. Friedl, "Glypican-1 stimulates Skp2 autoinduction loop and G1/S transition in endothelial cells," *J Biol Chem,* vol. 287, pp. 5898-909, Feb 17 2012.

[53] G. Raposo and W. Stoorvogel, "Extracellular vesicles: exosomes, microvesicles, and friends," *J Cell Biol,* vol. 200, pp. 373-83, Feb 18 2013.

[54] K. Denzer, M. J. Kleijmeer, H. F. Heijnen, W. Stoorvogel, and H. J. Geuze, "Exosome: from internal vesicle of the multivesicular body to intercellular signaling device," *J Cell Sci,* vol. 113 Pt 19, pp. 3365-74, Oct 2000.

[55] E. Cocucci and J. Meldolesi, "Ectosomes and exosomes: shedding the confusion between extracellular vesicles," *Trends Cell Biol,* vol. 25, pp. 364-72, Jun 2015.

[56] M. Verma, T. K. Lam, E. Hebert, and R. L. Divi, "Extracellular vesicles: potential applications in cancer diagnosis, prognosis, and epidemiology," *BMC Clin Pathol,* vol. 15, p. 6, 2015.

[57] M. F. Baietti, Z. Zhang, E. Mortier, A. Melchior, G. Degeest, A. Geeraerts, *et al.*, "Syndecan-syntenin-ALIX regulates the biogenesis of exosomes," *Nat Cell Biol,* vol. 14, pp. 677-85, Jul 2012.

[58] Z. Andreu and M. Yanez-Mo, "Tetraspanins in extracellular vesicle formation and function," *Front Immunol,* vol. 5, p. 442, 2014.

[59] B. J. Tauro, D. W. Greening, R. A. Mathias, S. Mathivanan, H. Ji, and R. J. Simpson, "Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids," *Mol Cell Proteomics,* vol. 12, pp. 587-98, Mar 2013.

[60] R. Gastpar, M. Gehrmann, M. A. Bausero, A. Asea, C. Gross, J. A. Schroeder, *et al.*, "Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells," *Cancer Res,* vol. 65, pp. 5238-47, Jun 15 2005.

[61] L. Camacho, P. Guerrero, and D. Marchetti, "MicroRNA and protein profiling of brain metastasis competent cell-derived exosomes," *PLoS One,* vol. 8, p. e73790, 2013.

[62] B. K. Thakur, H. Zhang, A. Becker, I. Matei, Y. Huang, B. Costa-Silva, *et al.*, "Double-stranded DNA in exosomes: a novel biomarker in cancer detection," *Cell Res,* vol. 24, pp. 766-9, Jun 2014.

[63] M. Record, K. Carayon, M. Poirot, and S. Silvente-Poirot, "Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies," *Biochim Biophys Acta,* vol. 1841, pp. 108-20, Jan 2014.
[64] M. F. Ribeiro, H. Zhu, R. W. Millard, and G. C. Fan, "Exosomes Function in Pro- and Anti-Angiogenesis," *Curr Angiogenes,* vol. 2, pp. 54-59, 2013.
[65] G. C. Fan, "Hypoxic exosomes promote angiogenesis," *Blood,* vol. 124, pp. 3669-70, Dec 11 2014.
[66] J. L. Hood, H. Pan, G. M. Lanza, S. A. Wickline, I. Consortium for Translational Research in Advanced, and Nanomedicine, "Paracrine induction of endothelium by tumor exosomes," *Lab Invest,* vol. 89, pp. 1317-28, Nov 2009.
[67] H. Yi, J. Ye, X. M. Yang, L. W. Zhang, Z. G. Zhang, and Y. P. Chen, "High-grade ovarian cancer secreting effective exosomes in tumor angiogenesis," *Int J Clin Exp Pathol,* vol. 8, pp. 5062-70, 2015.
[68] G. W. Hu, Q. Li, X. Niu, B. Hu, J. Liu, S. M. Zhou*, et al*., "Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells attenuate limb ischemia by promoting angiogenesis in mice," *Stem Cell Res Ther,* vol. 6, p. 10, 2015.
[69] A. Shabbir, A. Cox, L. Rodriguez-Menocal, M. Salgado, and E. Van Badiavas, "Mesenchymal Stem Cell Exosomes Induce Proliferation and Migration of Normal and Chronic Wound Fibroblasts, and Enhance Angiogenesis In Vitro," *Stem Cells Dev,* vol. 24, pp. 1635-47, Jul 15 2015.
[70] R. K. Jain, E. di Tomaso, D. G. Duda, J. S. Loeffler, A. G. Sorensen, and T. T. Batchelor, "Angiogenesis in brain tumours," *Nat Rev Neurosci,* vol. 8, pp. 610-22, Aug 2007.
[71] P. Kucharzewska, H. C. Christianson, J. E. Welch, K. J. Svensson, E. Fredlund, M. Ringner*, et al*., "Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development," *Proc Natl Acad Sci U S A,* vol. 110, pp. 7312-7, Apr 30 2013.
[72] J. Skog, T. Wurdinger, S. van Rijn, D. H. Meijer, L. Gainche, M. Sena-Esteves*, et al*., "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers," *Nat Cell Biol,* vol. 10, pp. 1470-6, Dec 2008.
[73] K. Al-Nedawi, B. Meehan, R. S. Kerbel, A. C. Allison, and J. Rak, "Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR," *Proc Natl Acad Sci U S A,* vol. 106, pp. 3794-9, Mar 10 2009.
[74] A. Bronisz, Y. Wang, M. O. Nowicki, P. Peruzzi, K. I. Ansari, D. Ogawa*, et al*., "Extracellular vesicles modulate the glioblastoma microenvironment via a tumor suppression signaling network directed by miR-1," *Cancer Res,* vol. 74, pp. 738-50, Feb 1 2014.
[75] U. Putz, J. Howitt, A. Doan, C. P. Goh, L. H. Low, J. Silke*, et al*., "The tumor suppressor PTEN is exported in exosomes and has phosphatase activity in recipient cells," *Sci Signal,* vol. 5, p. ra70, Sep 25 2012.

[76] J. de Vrij, S. L. Maas, K. M. Kwappenberg, R. Schnoor, A. Kleijn, L. Dekker, *et al.*, "Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells," *Int J Cancer,* vol. 137, pp. 1630-42, Oct 1 2015.

[77] N. Jetten, S. Verbruggen, M. J. Gijbels, M. J. Post, M. P. De Winther, and M. M. Donners, "Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo," *Angiogenesis,* vol. 17, pp. 109-18, Jan 2014.

[78] E. Zajac, B. Schweighofer, T. A. Kupriyanova, A. Juncker-Jensen, P. Minder, J. P. Quigley, *et al.*, "Angiogenic capacity of M1- and M2-polarized macrophages is determined by the levels of TIMP-1 complexed with their secreted proMMP-9," *Blood,* vol. 122, pp. 4054-67, Dec 12 2013.

[79] S. Ohno, M. Takanashi, K. Sudo, S. Ueda, A. Ishikawa, N. Matsuyama, *et al.*, "Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells," *Mol Ther,* vol. 21, pp. 185-91, Jan 2013.

[80] K. B. Johnsen, J. M. Gudbergsson, M. N. Skov, L. Pilgaard, T. Moos, and M. Duroux, "A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy," *Biochim Biophys Acta,* vol. 1846, pp. 75-87, Aug 2014.

[81] X. J. Tang, X. Y. Sun, K. M. Huang, L. Zhang, Z. S. Yang, D. D. Zou, *et al.*, "Therapeutic potential of CAR-T cell-derived exosomes: a cell-free modality for targeted cancer therapy," *Oncotarget,* Oct 19 2015.

[82] A. M. Rosca, D. M. Rayia, and R. Tutuianu, "Emerging Role of Stem Cells - Derived Exosomes as Valuable Tools for Cardiovascular Therapy," *Curr Stem Cell Res Ther,* Oct 26 2015.

[83] R. C. Lai, F. Arslan, M. M. Lee, N. S. Sze, A. Choo, T. S. Chen, *et al.*, "Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury," *Stem Cell Res,* vol. 4, pp. 214-22, May 2010.

[84] Y. Wang, L. Zhang, Y. Li, L. Chen, X. Wang, W. Guo, *et al.*, "Exosomes/microvesicles from induced pluripotent stem cells deliver cardioprotective miRNAs and prevent cardiomyocyte apoptosis in the ischemic myocardium," *Int J Cardiol,* vol. 192, pp. 61-9, Aug 1 2015.

[85] J. M. Vicencio, D. M. Yellon, V. Sivaraman, D. Das, C. Boi-Doku, S. Arjun, *et al.*, "Plasma exosomes protect the myocardium from ischemia-reperfusion injury," *J Am Coll Cardiol,* vol. 65, pp. 1525-36, Apr 21 2015.

[86] S. Liu, J. Sun, and Q. Lan, "Glioblastoma microvesicles promote endothelial cell proliferation through Akt/beta-catenin pathway," *Int J Clin Exp Pathol,* vol. 7, pp. 4857-66, 2014.

[87] M. G. Davies, "Criticial limb ischemia: epidemiology," *Methodist DeBakey cardiovascular journal,* vol. 8, pp. 10-4, 2012 2012.

[88] J. W. Olin, C. J. White, E. J. Armstrong, D. Kadian-Dodov, and W. R. Hiatt, "Peripheral Artery Disease Evolving Role of Exercise, Medical Therapy, and Endovascular Options," *Journal of the American College of Cardiology,* vol. 67, pp. 1338-1338, Mar 22 2016.

[89] M. Teraa, M. S. Conte, F. L. Moll, and M. C. Verhaar, "Critical Limb Ischemia: Current Trends and Future Directions," *Journal of the American Heart Association,* vol. 5, 2016 Feb 2016.

[90] J. J. Castronuovo, H. M. Adera, J. M. Smiell, and R. M. Price, "Skin perfusion pressure measurement is valuable in the diagnosis of critical limb ischemia," *Journal of Vascular Surgery,* vol. 26, pp. 629-637, Oct 1997.

[91] A. J. Comerota, R. C. Throm, K. A. Miller, T. Henry, N. Chronos, J. Laird, *et al.*, "Naked plasmid DNA encoding fibroblast growth factor type I for the treatment of end-stage unreconstructible lower extremity ischemia: Preliminary results of a phase I trial," *Journal of Vascular Surgery,* vol. 35, pp. 930-936, May 2002.

[92] U. Palmer-Kazen, D. Wariaro, F. W. Luo, and E. Wahlberg, "Vascular endothelial cell growth factor and fibroblast growth factor 2 expression in patients with critical limb ischemia," *Journal of Vascular Surgery,* vol. 39, pp. 621-628, Mar 2004.

[93] D. Shweiki, A. Itin, D. Soffer, and E. Keshet, "VASCULAR ENDOTHELIAL GROWTH-FACTOR INDUCED BY HYPOXIA MAY MEDIATE HYPOXIA-INITIATED ANGIOGENESIS," *Nature,* vol. 359, pp. 843-845, Oct 29 1992.

[94] H. Shigematsu, K. Yasuda, T. Iwai, T. Sasajima, S. Ishimaru, Y. Ohashi, *et al.*, "Randomized, double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical limb ischemia," *Gene Therapy,* vol. 17, pp. 1152-1161, Sep 2010.

[95] R. J. Powell, P. Goodney, F. O. Mendelsohn, E. K. Moen, B. H. Annex, and H. G. F. T. Investigators, "Safety and efficacy of patient specific intramuscular injection of HGF plasmid gene therapy on limb perfusion and wound healing in patients with ischemic lower extremity ulceration: Results of the HGF-0205 trial," *Journal of Vascular Surgery,* vol. 52, pp. 1525-1530, Dec 2010.

[96] S. H. Ko, A. Nauta, V. Wong, J. Glotzbach, G. C. Gurtner, and M. T. Longaker, "The Role of Stem Cells in Cutaneous Wound Healing: What Do We Really Know?," *Plastic and Reconstructive Surgery,* vol. 127, pp. 10S-20S, Jan 2011.

[97] G. Chamberlain, J. Fox, B. Ashton, and J. Middleton, "Concise review: Mesenchymal stem cells: Their phenotype, differentiation capacity, immunological features, and potential for homing," *Stem Cells,* vol. 25, pp. 2739-2749, Nov 2007.

[98] T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. vanderZee, T. Li, *et al.*, "Isolation of putative progenitor endothelial cells for angiogenesis," *Science,* vol. 275, pp. 964-967, Feb 14 1997.

[99] M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, *et al.*, "Multilineage potential of adult human mesenchymal stem cells," *Science,* vol. 284, pp. 143-147, Apr 2 1999.

[100] M. Murakami, T. Kambe, S. Shimbara, S. Yamamoto, H. Kuwata, and I. Kudo, "Functional association of type IIA secretory phospholipase A, with the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan in the

cyclooxygenase-2-mediated delayed prostanoid-biosynthetic pathway," *Journal of Biological Chemistry,* vol. 274, pp. 29927-29936, Oct 15 1999.

[101] E. Marois, A. Mahmoud, and S. Eaton, "The endocytic pathway and formation of the Wingless morphogen gradient," *Development,* vol. 133, pp. 307-317, Jan 2006.

[102] A. Gallet, L. Staccini-Lavenant, and P. P. Therond, "Cellular trafficking of the glypican dally-like is required for full-strength Hedgehog signaling and Wingless transcytosis," *Developmental Cell,* vol. 14, pp. 712-725, May 2008.

[103] K. Beckett, X. Franch-Marro, and J.-P. Vincent, "Glypican-mediated endocytosis of Hedgehog has opposite effects in flies and mice," *Trends in Cell Biology,* vol. 18, pp. 360-363, Aug 2008.

[104] H. C. Christianson, K. J. Svensson, T. H. van Kuppevelt, J.-P. Li, and M. Belting, "Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 110, pp. 17380-17385, Oct 22 2013.

[105] J. Ledin, W. Staatz, J. P. Li, M. Gotte, S. Selleck, L. Kjellen, *et al.*, "Heparan sulfate structure in mice with genetically modified heparan sulfate production," *Journal of Biological Chemistry,* vol. 279, pp. 42732-42741, Oct 2004.

[106] J. Kreuger, L. Perez, A. J. Giraldez, and S. M. Cohen, "Opposing activities of dally-like glypican at high and low levels of wingless morphogen activity," *Developmental Cell,* vol. 7, pp. 503-512, Oct 2004.

[107] J. Gutierrez, D. Cabrera, and E. Brandan, "Glypican-1 regulates myoblast response to HGF via Met in a lipid raft-dependent mechanism: effect on migration of skeletal muscle precursor cells," *Skeletal Muscle,* vol. 4, Feb 12 2014.

[108] A. Chamorro-Jorganes, E. Araldi, N. Rotllan, D. Cirera-Salinas, and Y. Suarez, "Autoregulation of glypican-1 by intronic microRNA-149 fine tunes the angiogenic response to FGF2 in human endothelial cells," *Journal of Cell Science,* vol. 127, pp. 1169-1178, Mar 15 2014.

[109] P. P. Dwivedi, R. H. Grose, J. Filmus, C. S. T. Hii, C. J. Xian, P. J. Anderson, *et al.*, "Regulation of bone morphogenetic protein signalling and cranial osteogenesis by Gpc1 and Gpc3," *Bone,* vol. 55, pp. 367-376, Aug 2013.

[110] J. Kleeff, T. Ishiwata, A. Kumbasar, H. Friess, M. W. Buchler, A. D. Lander, *et al.*, "The cell-surface heparan sulfate proteoglycan glypican-1 regulates growth factor action in pancreatic carcinoma cells and is overexpressed in human pancreatic cancer," *Journal of Clinical Investigation,* vol. 102, pp. 1662-1673, Nov 1 1998.

[111] G. Su, K. Meyer, C. D. Nandini, D. H. Qiao, S. Salamat, and A. Friedl, "Glypican-1 is frequently overexpressed in human gliomas and enhances FGF-2 signaling in glioma cells," *American Journal of Pathology,* vol. 168, pp. 2014-2026, Jun 2006.

[112] D. H. Qiao, K. Meyer, C. Mundhenke, S. A. Drew, and A. Friedl, "Heparan sulfate proteoglycans as regulators of fibroblast growth factor-2 signaling in brain

endothelial cells," *Journal of Biological Chemistry,* vol. 278, pp. 16045-16053, May 2 2003.

[113] A. D. Theocharis, S. S. Skandalis, G. N. Tzanakakis, and N. K. Karamanos, "Proteoglycans in health and disease: novel roles for proteoglycans in malignancy and their pharmacological targeting," *Febs Journal,* vol. 277, pp. 3904-3923, Oct 2010.

[114] D. Qiao, K. Meyer, and A. Friedl, "Glypican 1 Stimulates S Phase Entry and DNA Replication in Human Glioma Cells and Normal Astrocytes," *Molecular and Cellular Biology,* vol. 33, pp. 4408-4421, Nov 2013.

[115] S. A. Melo, L. B. Luecke, C. Kahlert, A. F. Fernandez, S. T. Gammon, J. Kaye*, et al.*, "Glypican-1 identifies cancer exosomes and detects early pancreatic cancer," *Nature,* vol. 523, pp. 177-U82, Jul 9 2015.

[116] S. Gengrinovitch, B. Berman, G. David, L. Witte, G. Neufeld, and D. Ron, "Glypican-1 is a VEGF(165) binding proteoglycan that acts as an extracellular chaperone for VEGF(165)," *Journal of Biological Chemistry,* vol. 274, pp. 10816-10822, Apr 16 1999.

[117] Y.-H. L. Jen, M. Musacchio, and A. D. Lander, "Glypican-1 controls brain size through regulation of fibroblast growth factor signaling in early neurogenesis," *Neural Development,* vol. 4, Sep 4 2009.

[118] T. Aikawa, C. A. Whipple, M. E. Lopez, J. Gunn, A. Young, A. D. Lander*, et al.*, "Glypican-1 modulates the angiogenic and metastatic potential of human and mouse cancer cells," *Journal of Clinical Investigation,* vol. 118, pp. 89-99, Jan 2008.

[119] E. Cervio, L. Barile, T. Moccetti, and G. Vassalli, "Exosomes for Intramyocardial Intercellular Communication," *Stem Cells International,* 2015 2015.

[120] G. Raposo and W. Stoorvogel, "Extracellular vesicles: Exosomes, microvesicles, and friends," *Journal of Cell Biology,* vol. 200, pp. 373-383, Feb 2013.

[121] M. Iero, R. Valenti, V. Huber, P. Filipazzi, G. Parmiani, S. Fais*, et al.*, "Tumour-released exosomes and their implications in cancer immunity," *Cell Death and Differentiation,* vol. 15, pp. 80-88, Jan 2008.

[122] D. D. Taylor and C. Gercel-Taylor, "Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments," *Seminars in Immunopathology,* vol. 33, pp. 441-454, Sep 2011.

[123] S. Brem, J. Folkman, and R. Cotran, "TUMOR ANGIOGENESIS - QUANTITATIVE METHOD FOR HISTOLOGIC GRADING," *Journal of the National Cancer Institute,* vol. 48, pp. 347-&, 1972 1972.

[124] J. Godlewski, A. M. Krichevsky, M. D. Johnson, E. A. Chiocca, and A. Bronisz, "Belonging to a network-microRNAs, extracellular vesicles, and the glioblastoma microenvironment," *Neuro-Oncology,* vol. 17, pp. 652-662, May 2015.

[125] S. Mukhopadhyay and A. D. Linstedt, "Identification of a gain-of-function mutation in a Golgi P-type ATPase that enhances Mn2+ efflux and protects against toxicity," *Proc Natl Acad Sci U S A,* vol. 108, pp. 858-63, Jan 11 2011.

[126] W. Chen, Y. Feng, D. Chen, and A. Wandinger-Ness, "Rab11 is required for trans-golgi network-to-plasma membrane transport and a preferential target for GDP dissociation inhibitor," *Mol Biol Cell,* vol. 9, pp. 3241-57, Nov 1998.

[127] E. Jang, H. Albadawi, M. T. Watkins, E. R. Edelman, and A. B. Baker, "Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle," *Proc Natl Acad Sci U S A,* vol. 109, pp. 1679-84, Jan 31 2012.

[128] R. Cao, A. Eriksson, H. Kubo, K. Alitalo, Y. Cao, and J. Thyberg, "Comparative evaluation of FGF-2-, VEGF-A-, and VEGF-C-induced angiogenesis, lymphangiogenesis, vascular fenestrations, and permeability," *Circ Res,* vol. 94, pp. 664-70, Mar 19 2004.

[129] A. Elfenbein, A. Lanahan, T. X. Zhou, A. Yamasaki, E. Tkachenko, M. Matsuda, *et al*., "Syndecan 4 regulates FGFR1 signaling in endothelial cells by directing macropinocytosis," *Sci Signal,* vol. 5, p. ra36, May 8 2012.

[130] K. Mani, F. Cheng, and L. A. Fransson, "Constitutive and vitamin C-induced, NO-catalyzed release of heparan sulfate from recycling glypican-1 in late endosomes," *Glycobiology,* vol. 16, pp. 1251-61, Dec 2006.

[131] S. Jean, A. Mikryukov, M. G. Tremblay, J. Baril, F. Guillou, S. Bellenfant, *et al*., "Extended-synaptotagmin-2 mediates FGF receptor endocytosis and ERK activation in vivo," *Dev Cell,* vol. 19, pp. 426-39, Sep 14 2010.

[132] P. L. Voyvodic, D. Min, R. Liu, E. Williams, V. Chitalia, A. K. Dunn, *et al*., "Loss of syndecan-1 induces a pro-inflammatory phenotype in endothelial cells with a dysregulated response to atheroprotective flow," *J Biol Chem,* vol. 289, pp. 9547-59, Apr 4 2014.

[133] R. J. Laham, M. Rezaee, M. Post, D. Novicki, F. W. Sellke, J. D. Pearlman, *et al*., "Intrapericardial delivery of fibroblast growth factor-2 induces neovascularization in a porcine model of chronic myocardial ischemia," *J Pharmacol Exp Ther,* vol. 292, pp. 795-802, Feb 2000.

[134] R. Baffour, J. Berman, J. L. Garb, S. W. Rhee, J. Kaufman, and P. Friedmann, "Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor," *J Vasc Surg,* vol. 16, pp. 181-91, Aug 1992.

[135] J. Gutierrez and E. Brandan, "A novel mechanism of sequestering fibroblast growth factor 2 by glypican in lipid rafts, allowing skeletal muscle differentiation," *Mol Cell Biol,* vol. 30, pp. 1634-49, Apr 2010.

[136] J. Shin, D. C. McFarland, and S. G. Velleman, "Heparan sulfate proteoglycans, syndecan-4 and glypican-1, differentially regulate myogenic regulatory transcription factors and paired box 7 expression during turkey satellite cell myogenesis: implications for muscle growth," *Poult Sci,* vol. 91, pp. 201-7, Jan 2012.

[137] D. P. Slovut and T. M. Sullivan, "Critical limb ischemia: medical and surgical management," *Vasc Med,* vol. 13, pp. 281-91, Aug 2008.

[138] M. B. Sherman, R. H. Guenther, F. Tama, T. L. Sit, C. L. Brooks, A. M. Mikhailov, *et al.*, "Removal of divalent cations induces structural transitions in red clover necrotic mosaic virus, revealing a potential mechanism for RNA release," *J Virol,* vol. 80, pp. 10395-406, Nov 2006.
[139] C. Trapnell, L. Pachter, and S. L. Salzberg, "TopHat: discovering splice junctions with RNA-Seq," *Bioinformatics,* vol. 25, pp. 1105-1111, May 1 2009.
[140] S. Anders, P. T. Pyl, and W. Huber, "HTSeq-a Python framework to work with high-throughput sequencing data," *Bioinformatics,* vol. 31, pp. 166-169, Jan 15 2015.
[141] S. Anders and W. Huber, "Differential expression analysis for sequence count data," *Genome Biology,* vol. 11, 2010 2010.
[142] S. Nicoli, C.-P. Knyphausen, L. J. Zhu, A. Lakshmanan, and N. D. Lawson, "miR-221 Is Required for Endothelial Tip Cell Behaviors during Vascular Development," *Developmental Cell,* vol. 22, pp. 418-429, Feb 14 2012.
[143] L. Poliseno, A. Tuccoli, L. Mariani, M. Evangelista, L. Citti, K. Woods, *et al.*, "MicroRNAs modulate the angiogenic properties of HLTVECs," *Blood,* vol. 108, pp. 3068-3071, Nov 2006.
[144] J. Novakova, O. Slaby, R. Vyzula, and J. Michalek, "MicroRNA involvement in glioblastoma pathogenesis," *Biochemical and Biophysical Research Communications,* vol. 386, pp. 1-5, Aug 14 2009.
[145] S. He, R. Lai, D. Chen, W. Yan, Z. Zhang, Z. Liu, *et al.*, "Downregulation of miR-221 Inhibits Cell Migration and Invasion through Targeting Methyl-CpG Binding Domain Protein 2 in Human Oral Squamous Cell Carcinoma Cells," *BioMed research international,* vol. 2015, pp. 751672-751672, 2015 2015.
[146] X. Wang, W. Huang, G. Liu, W. Cai, R. W. Millard, Y. Wang, *et al.*, "Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into endothelial cells," *Journal of Molecular and Cellular Cardiology,* vol. 74, pp. 139-150, Sep 2014.
[147] X. H. Wang, R. Z. Qian, W. Zhang, S. F. Chen, H. M. Jin, and R. M. Hu, "MicroRNA-320 EXPRESSION IN MYOCARDIAL MICROVASCULAR ENDOTHELIAL CELLS AND ITS RELATIONSHIP WITH INSULIN-LIKE GROWTH FACTOR-1 IN TYPE 2 DIABETIC RATS," *Clinical and Experimental Pharmacology and Physiology,* vol. 36, pp. 181-188, Feb 2009.
[148] S. K. Hermansen, B. S. Nielsen, C. Aaberg-Jessen, and B. W. Kristensen, "miR-21 Is Linked to Glioma Angiogenesis: A Co-Localization Study," *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,* vol. 64, pp. 138-48, 2016-Feb 2016.
[149] Y. Liu, F. Luo, B. Wang, H. Li, Y. Xu, X. Liu, *et al.*, "STAT3-regulated exosomal miR-21 promotes angiogenesis and is involved in neoplastic processes of transformed human bronchial epithelial cells," *Cancer Letters,* vol. 370, pp. 125-135, 2016 2016.

[150] A. L. Kasinski and F. J. Slack, "MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy," *Nature Reviews Cancer,* vol. 11, pp. 849-864, Dec 2011.
[151] M. Singh, N. Garg, C. Venugopal, R. Hallett, T. Tokar, N. McFarlane, *et al.*, "STAT3 pathway regulates lung-derived brain metastasis initiating cell capacity through miR-21 activation," *Oncotarget,* vol. 6, pp. 27461-27477, Sep 29 2015.
[152] Z. Wang, H. Yang, and L. Ren, "MiR-21 promoted proliferation and migration in hepatocellular carcinoma through negative regulation of Navigator-3," *Biochemical and Biophysical Research Communications,* vol. 464, pp. 1228-1234, Sep 4 2015.
[153] J. Fiedler, V. Jazbutyte, B. C. Kirchmaier, S. K. Gupta, J. Lorenzen, D. Hartmann, *et al.*, "MicroRNA-24 Regulates Vascularity After Myocardial Infarction," *Circulation,* vol. 124, pp. 720-U178, Aug 9 2011.
[154] R. Liu, H. Zhang, X. Wang, L. Zhou, H. Li, T. Deng, *et al.*, "The miR-24-Bim pathway promotes tumor growth and angiogenesis in pancreatic carcinoma," *Oncotarget,* vol. 6, pp. 43831-43842, Dec 22 2015.
[155] H. Zhang, J. Duan, Y. Qu, T. Deng, R. Liu, L. Zhang, *et al.*, "Onco-miR-24 regulates cell growth and apoptosis by targeting BCL2L11 in gastric cancer," *Protein & cell,* vol. 7, pp. 141-51, 2016-Feb 2016.
[156] E. Jusufovic, M. Rijavec, D. Keser, P. Korosec, E. Sodja, E. Iljazovic, *et al.*, "let-7b and miR-126 Are Down-Regulated in Tumor Tissue and Correlate with Microvessel Density and Survival Outcomes in Non-Small-Cell Lung Cancer," *Plos One,* vol. 7, Sep 24 2012.
[157] Y. Tian, S. Hao, M. Ye, A. Zhang, Y. Nan, G. Wang, *et al.*, "MicroRNAs let-7b/i suppress human glioma cell invasion and migration by targeting IKBKE directly," *Biochemical and Biophysical Research Communications,* vol. 458, pp. 307-312, Mar 6 2015.
[158] N. Yamada, N. Tsujimura, M. Kumazaki, H. Shinohara, K. Taniguchi, Y. Nakagawa, *et al.*, "Colorectal cancer cell-derived microvesicles containing microRNA-1246 promote angiogenesis by activating Smad 1/5/8 signaling elicited by PML down-regulation in endothelial cells," *Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms,* vol. 1839, pp. 1256-1272, Nov 2014.
[159] S. Wang, Y. Zeng, J.-M. Zhou, S.-L. Nie, Q. Peng, J. Gong, *et al.*, "MicroRNA-1246 promotes growth and metastasis of colorectal cancer cells involving CCNG2 reduction," *Molecular Medicine Reports,* vol. 13, pp. 273-280, Jan 2016.
[160] X.-R. Wang, H. Luo, H.-L. Li, L. Cao, X.-F. Wang, W. Yan, *et al.*, "Overexpressed let-7a inhibits glioma cell malignancy by directly targeting K-ras, independently of PTEN," *Neuro-Oncology,* vol. 15, pp. 1491-1501, Nov 2013.
[161] Z. Chen, T.-C. Lai, Y.-H. Jan, F.-M. Lin, W.-C. Wang, H. Xiao, *et al.*, "Hypoxia-responsive miRNAs target argonaute 1 to promote angiogenesis," *Journal of Clinical Investigation,* vol. 123, pp. 1057-1067, Mar 2013.

[162] G. Martello, A. Rosato, F. Ferrari, A. Manfrin, M. Cordenonsi, S. Dupont, *et al*., "A MicroRNA Targeting Dicer for Metastasis Control," *Cell,* vol. 141, pp. 1195-U176, Jun 25 2010.
[163] X. Fu, W. Zhang, Y. Su, L. Lu, D. Wang, and H. Wang, "MicroRNA-103 suppresses tumor cell proliferation by targeting PDCD10 in prostate cancer," *The Prostate,* vol. 76, pp. 543-51, 2016 May (Epub 2016 Jan 2016.
[164] Y. Li, L. Mao, Y. Gao, S. Baral, Y. Zhou, and B. Hu, "MicroRNA-107 contributes to post-stroke angiogenesis by targeting Dicer-1," *Scientific Reports,* vol. 5, Aug 21 2015.
[165] L. Chen, Z.-y. Li, S.-y. Xu, X.-j. Zhang, Y. Zhang, K. Luo, *et al*., "Upregulation of miR-107 Inhibits Glioma Angiogenesis and VEGF Expression," *Cellular and Molecular Neurobiology,* vol. 36, pp. 113-120, Jan 2016.
[166] G. Z. Wang, Y. Zhao, and Y. R. Zheng, "miR-122/Wnt/beta-catenin regulatory circuitry sustains glioma progression," *Tumor Biology,* vol. 35, pp. 8565-8572, Sep 2014.
[167] S. Bai, M. W. Nasser, B. Wang, S.-H. Hsu, J. Datta, H. Kutay, *et al*., "MicroRNA-122 Inhibits Tumorigenic Properties of Hepatocellular Carcinoma Cells and Sensitizes These Cells to Sorafenib," *Journal of Biological Chemistry,* vol. 284, pp. 32015-32027, Nov 13 2009.
[168] H. T. Li, H. Zhang, Y. Chen, X. F. Liu, and J. Qian, "MiR-423-3p Enhances Cell Growth Through Inhibition of p21Cip1/Waf1 in Colorectal Cancer," *Cellular Physiology and Biochemistry,* vol. 37, pp. 1044-1054, Sep 2015.
[169] J. Zhang, X. Gong, K. Tian, D. Chen, J. Sun, G. Wang, *et al*., "miR-25 promotes glioma cell proliferation by targeting CDKN1C," *Biomedicine & Pharmacotherapy,* vol. 71, pp. 7-14, Apr 2015.
[170] H.-H. Kwok, L.-S. Chan, P.-Y. Poon, P. Y.-K. Yue, and R. N.-S. Wong, "Ginsenoside-Rg(1) induces angiogenesis by the inverse regulation of MET tyrosine kinase receptor expression through miR-23a," *Toxicology and Applied Pharmacology,* vol. 287, pp. 276-283, Sep 15 2015.
[171] S. Lian, R. Shi, T. Bai, Y. Liu, W. Miao, H. Wang, *et al*., "Anti-miRNA-23a Oligonucleotide Suppresses Glioma Cells Growth by Targeting Apoptotic Protease Activating Factor-1," *Current Pharmaceutical Design,* vol. 19, pp. 6382-6389, Oct 2013.
[172] J. K. Lee, S. R. Park, B. K. Jung, Y. K. Jeon, Y. S. Lee, M. K. Kim, *et al*., "Exosomes Derived from Mesenchymal Stem Cells Suppress Angiogenesis by Down-Regulating VEGF Expression in Breast Cancer Cells," *Plos One,* vol. 8, p. 11, Dec 2013.
[173] E. Salerno, B. J. Scaglione, F. D. Coffman, B. D. Brown, A. Baccarini, H. Fernandes, *et al*., "Correcting miR-15a/16 genetic defect in New Zealand Black mouse model of CLL enhances drug sensitivity," *Molecular Cancer Therapeutics,* vol. 8, pp. 2684-2692, Sep 2009.
[174] W.-L. Wu, W.-Y. Wang, W.-Q. Yao, and G.-D. Li, "Suppressive effects of microRNA-16 on the proliferation, invasion and metastasis of hepatocellular

carcinoma cells," *International Journal of Molecular Medicine,* vol. 36, pp. 1713-1719, Dec 2015.
[175] S. Yan, X. Han, H. Xue, P. Zhang, X. Guo, T. Li, *et al.*, "Let-7f Inhibits Glioma Cell Proliferation, Migration, and Invasion by Targeting Periostin," *Journal of Cellular Biochemistry,* vol. 116, pp. 1680-1692, Aug 2015.
[176] S. Liang, L. He, X. Zhao, Y. Miao, Y. Gu, C. Guo, *et al.*, "MicroRNA Let-7f Inhibits Tumor Invasion and Metastasis by Targeting MYH9 in Human Gastric Cancer," *Plos One,* vol. 6, Apr 18 2011.
[177] H. T. Wang, F. Cao, X. Li, H. Miao, J. F. E, J. J. Xing, *et al.*, "miR-320b suppresses cell proliferation by targeting c-Myc in human colorectal cancer cells," *Bmc Cancer,* vol. 15, p. 9, Oct 2015.
[178] H.-T. Liu, A.-Y. Xing, X. Chen, R.-R. Ma, Y.-W. Wang, D.-B. Shi, *et al.*, "MicroRNA-27b, microRNA-101 and microRNA-128 inhibit angiogenesis by down-regulating vascular endothelial growth factor C expression in gastric cancers," *Oncotarget,* vol. 6, pp. 37467-37479, Nov 10 2015.
[179] L. Chen, H. Li, L. Han, K. Zhang, G. Wang, Y. Wang, *et al.*, "Expression and function of miR-27b in human glioma," *Oncology Reports,* vol. 26, pp. 1617-1621, Dec 2011.
[180] S. Zhang, F. Liu, X. Ma, J. Huang, J. Yang, X. Yin, *et al.*, "Elevation of miR-27b by HPV16 E7 inhibits PPAR gamma expression and promotes proliferation and invasion in cervical carcinoma cells," *International Journal of Oncology,* vol. 47, pp. 1759-1766, Nov 2015.
[181] D. Y. Lee, Z. Deng, C.-H. Wang, and B. B. Yang, "MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, pp. 20350-20355, Dec 18 2007.
[182] Y. Ma, S. Yu, W. Zhao, Z. Lu, and J. Chen, "miR-27a regulates the growth, colony formation and migration of pancreatic cancer cells by targeting Sprouty2," *Cancer Letters,* vol. 298, pp. 150-158, Dec 8 2010.
[183] X.-F. Shi, H. Wang, F.-J. Xiao, Y. Yin, Q.-Q. Xu, R.-L. Ge, *et al.*, "MiRNA-486 regulates angiogenic activity and survival of mesenchymal stem cells under hypoxia through modulating Akt signal," *Biochemical and Biophysical Research Communications,* vol. 470, pp. 670-677, Feb 12 2016.
[184] K. Wu, Y. Yang, J. Zhao, and S. Zhao, "BAG3-mediated miRNA let-7g and let-7i inhibit proliferation and enhance apoptosis of human esophageal carcinoma cells by targeting the drug transporter ABCC10," *Cancer Letters,* vol. 371, pp. 125-133, 2016 2016.
[185] L. Fang, W. W. Du, W. Yang, Z. J. Rutnam, C. Peng, H. Li, *et al.*, "MiR-93 enhances angiogenesis and metastasis by targeting LATS2," *Cell Cycle,* vol. 11, pp. 4352-4365, Dec 1 2012.
[186] G. T. Liu, Y. L. Huang, H. E. Tzeng, C. H. Tsai, S. W. Wang, and C. H. Tang, "CCL5 promotes vascular endothelial growth factor expression and induces

angiogenesis by down-regulating miR-199a in human chondrosarcoma cells," *Cancer Letters,* vol. 357, pp. 476-487, Feb 2015.

[187] J. Zeng, L. Chen, B. Chen, K. Z. Lu, K. Belguise, X. B. Wang, *et al.*, "MicroRNA-199a-5p Regulates the Proliferation of Pulmonary Microvascular Endothelial Cells in Hepatopulmonary Syndrome," *Cellular Physiology and Biochemistry,* vol. 37, pp. 1289-1300, 2015.

[188] T. Chen, A. Margariti, S. Kelaini, A. Cochrane, S. T. Guha, Y. Hu, *et al.*, "MicroRNA-199b Modulates Vascular Cell Fate During iPS Cell Differentiation by Targeting the Notch Ligand Jagged1 and Enhancing VEGF Signaling," *Stem Cells,* vol. 33, pp. 1405-1418, May 2015.

[189] H. Zeng, Z. Zhang, X. Dai, Y. Chen, J. Ye, and Z. Jin, "Increased Expression of microRNA-199b-5p Associates with Poor Prognosis Through Promoting Cell Proliferation, Invasion and Migration Abilities of Human Osteosarcoma," *Pathology oncology research : POR,* vol. 22, pp. 253-60, 2016-Apr 2016.

[190] Q. Jiang, M. Lagos-Quintana, D. Liu, Y. Shi, C. Helker, W. Herzog, *et al.*, "miR-30a Regulates Endothelial Tip Cell Formation and Arteriolar Branching," *Hypertension,* vol. 62, pp. 592-598, Sep 2013.

[191] J. R. Dobson, H. Taipaleenmaeki, Y. J. Hu, D. Hong, A. J. van Wijnen, J. L. Stein, *et al.*, "hsa-mir-30c promotes the invasive phenotype of metastatic breast cancer cells by targeting NOV/CCN3," *Cancer Cell International,* vol. 14, p. 14, Aug 2014.

[192] R. He, L. Yang, X. Lin, X. Chen, X. Lin, F. Wei, *et al.*, "MiR-30a-5p suppresses cell growth and enhances apoptosis of hepatocellular carcinoma cells via targeting AEG-1," *International Journal of Clinical and Experimental Pathology,* vol. 8, pp. 15632-15641, 2015 2015.

[193] F. Yang, W. Wang, C. Zhou, W. Xi, L. Yuan, X. Chen, *et al.*, "MiR-221/222 promote human glioma cell invasion and angiogenesis by targeting TIMP2," *Tumor Biology,* vol. 36, pp. 3763-3773, May 2015.

[194] E. Jang, H. Albadawi, M. T. Watkins, E. R. Edelman, and A. B. Baker, "Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 109, pp. 1679-1684, Jan 31 2012.

[195] S. Mukhopadhyay and A. D. Linstedt, "Identification of a gain-of-function mutation in a Golgi P-type ATPase that enhances Mn2+ efflux and protects against toxicity," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 108, pp. 858-863, Jan 11 2011.